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Delayed adsorption on slowly accessible stationary phase surfaces in porous liquid chromatographic column packings

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Abstract

Variation in the fractional area of the tailing portion of elution peak in HPLC was analysed over a wide range of mobile phase flow-rates. A slow adsorption–desorption process was found to contribute to solute retention only in the range of column hold-up times by at least two orders of magnitude longer than those usually used in analytical or preparative separation. The apparent first-order rate constant was evaluated to be of the order of 10^3–10^4 s^{-1} for the slow adsorption and the dependence of the rate constant on column temperature and mobile phase composition was analysed. The behaviour of the apparent rate constant of the slow adsorption could be explained as being due to slow diffusion by which solute molecules are transferred to the stationary phase surface. The stationary phase surface causing the slow adsorption may be considered thermodynamically equivalent with the rest of the stationary phase surface contained in the column and to which low-molecular-mass samples have geometrical access requiring considerable time owing to slow pore diffusion.

Keywords: Adsorption; Stationary phases, LC; Kinetic studies; Peak shape; Thermodynamic parameters; Tri-anisoyldeoxycytidine

1. Introduction

Chromatographic efficiency and performance are determined primarily by the system matrix used. A porous microparticulate column packing provides an excellent matrix for most applications of liquid chromatography (LC). This type of packing is characterized by a large specific surface area to increase the amount of adsorbed solute molecules within the linear region of the adsorption isotherm and minimized particle size to diminish solute dispersion in the mobile phase flow stream. The use of such material should make it possible to maximize sample loadability and resolution for a given chromatographic separation.

A porous matrix is not always advantageous for chromatographic separation, particularly in the case of macromolecular compounds. The slow diffusion of protein molecules in the pores of the packing leads to low efficiency and poor recovery in chromatographic separations in affinity [1], size exclusion [2] or reversed-phase [3] modes. In affinity chromatography [1], injected solute molecules consisting of a single protein

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species split into two elution peaks, one of which elutes in the hold-up volume of the mobile phase and the other is retained owing to solute adsorption on affinity ligands on the stationary phase surface. The former peak represents the solute molecules that failed to be adsorbed on the ligands owing to slow adsorption kinetics. One explanation for this is that the slow diffusion of macromolecules in a porous matrix prolongs the time required to reach the stationary phase surface on which the solute molecules are adsorbed by ligands. This is why non-porous adsorbents are recommended for the chromatographic separation of macromolecular samples [4], although the high loadability possible by porous material would be compromised.

Delayed adsorption due to pore diffusion may also occur in the case of smaller solute molecules injected into a porous LC column. The pore size distribution may be significant in any porous packing [5]. Most pores can be easily and rapidly penetrated by low-molecular-mass solutes and solvents and the pore walls are accessible as stationary phase surfaces, but adsorption on some walls may be hindered by slow diffusion in narrow regions of the pore network [5] or deep within the interior of (or in the "core" region of) porous particles of the packing [6]. Some of the adsorbent surface may thus be inaccessible to solutes carried rapidly by the mobile phase flow, with consequently insufficient time for slow pore diffusion.

Nevertheless, since adsorption on such a portion of the surface is not physically (or geometrically) prevented but merely delayed, that area would contribute to retention of sample solutes if the mobile phase flow-rate is reduced and the solute residence time in the mobile phase (i.e., column hold-up time) is prolonged. At an intermediate flow-rate, only a fraction of each of the injected solutes may be adsorbed on this limited area of the surface. The surface is called a "slowly accessible surface" in this paper.

It should be noted that slow pore diffusion also delays the process in the opposite direction, i.e., desorption of solute molecules to the mobile phase stream. If the mean time required for solute molecules to be desorbed compares with the degree of peak dispersion, the fraction of solutes adsorbed on a "slowly accessible surface" gives rise to a "tailing" portion of the peak.

The peak shape change observed with varying flow-rate of the mobile phase in liquid–solid chromatography (LSC) was previously shown [7] to be consistent with that expected on the assumption of a slow adsorption–desorption process. The area of the tailing portion of elution peak was mathematically evaluated by subtracting the Gaussian function best fitted to the leading part of each experimental LSC peak obtained for a highly diluted sample solute. The fractional area of the tailing portion increased gradually with decrease in the mobile phase flow-rate. According to the stochastic theory of slow adsorption–desorption process [8,9], the decrease in the fractional area of the Gaussian portion accompanying the "growth" of the tailing portion is related to the rate constant of slow adsorption process.

The present study was conducted to examine the rate constant of slow adsorption processes causing peak tailing in LSC. In this regard, the following assumptions were made:

(1) Growth of the tailing portion of the peak is a result of an increase in the number of solute molecules participating in the slow adsorption–desorption process during passage through the column.

(2) The logarithm of the fractional area of the Gaussian portion of peak decreases linearly with hold-up time of the column with the slope of the apparent first-order rate constant of the slow adsorption process.

(3) The effect of a non-linear adsorption isotherm at high solute dilution on the peak shape is represented by the asymmetry coefficient, independent of the hold-up time of the column.

(4) Significant contributions to the mean adsorption time, the inverse of the apparent rate constant, may come from diffusion and adsorption steps.

(5) The contribution of diffusion is due to the existence of pores intervening between the bulk

mobile phase stream and the stationary phase surface.

(6) The contribution of adsorption is explained by the solute–solvent exchange on the stationary phase surface in equilibrium with the mobile phase solvent, which is essentially the same mechanism as that usually assumed for a normal fast adsorption process.

(7) Adsorption of water from the mobile phase is assumed to inactivate adsorption sites on the silica gel surface with respect to solute adsorption, and the surface density of the remaining active sites may determine the kinetics of solute adsorption.

(8) Effective thermodynamic equilibrium is assumed to exist between the thermodynamic activities of the solvent components in the mobile phase and those in the first adsorbed layer. The activity coefficients of solvents in the first adsorbed layer are assumed to be unity because of the localized adsorption of solvents on adsorption sites of silica gel surface.

2. Experimental

2.1. Chemicals

Solvents were obtained from Wako (Osaka, Japan). Water was distilled and deionized. Deoxynucleoside derivatives as sample solutes were prepared in our laboratory.

2.2. Chromatographic experiments

Chromatographic experiments were carried out on a Model 5021 chromatograph (Varian, Palo Alto, CA, USA). The columns used were 250 mm × 4 mm I.D., packed with LiChrosorb Si 60 (5 μm) or LiChrospher Si 1000 (10 μm) (Merck, Darmstadt, Germany). A syringe-type chromatographic pump (LCP-350; Japan Spectroscopic, Tokyo, Japan) was used.

Chromatographic data acquisition, curve fitting and calculation of the fractional area of the elution peak were carried out as described previously [7].

3. Theory

3.1. Peak shape analysis for evaluating the apparent rate constant of slow adsorption process

The fundamental theory used in peak shape analysis is based on the stochastic theoretical model developed by Giddings [8,9] to simulate the chromatographic process which gives rise to the peak tailing phenomenon. The chromatographic mechanism presumed in this model consists of fast and slow repeated adsorption–desorption processes. Each of these processes can be independently described by stochastic theory. The elution profile of a solute, which has experienced an adsorption–desorption process, may be expressed by an equation derived from this theory (the stochastic equation shown in Fig. 1).

According to the stochastic equation, the elution profile should undergo transformation by altering the residence time of the solute in the mobile phase, from a highly asymmetric profile with long tailing for a relatively short residence time to a symmetric Gaussian-form profile for a much longer residence time. The latter Gaussian-form profile is the theoretical asymptotic form of the elution peak found only when an infinitely large number of adsorption–desorption steps are taken by solute molecules during passage through the column.

For a fast adsorption–desorption process as considered to occur in actual LSC systems as a "normal" chromatographic process, even the highest mobile phase flow-rate may make possible a sufficiently long mobile-phase residence time and a sufficient number of adsorption–desorption steps to find Gaussian-form elution profiles (as seen in Fig. 1A).

If a slow adsorption–desorption process occurs along with a fast process, we may observe the transformation of the elution profile within a range of mobile-phase residence times. In this study, such a time range is referred to as the "peak-transforming mobile-phase residence time region". According to the simulation based on

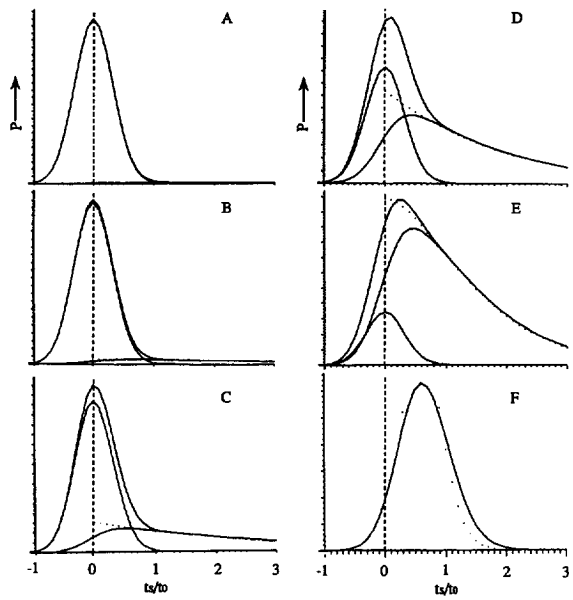

Fig. 1. Simulation of the transformation of a chromatographic peak profile in the presence of a slow adsorption–desorption process by means of the stochastic equation proposed by Giddings [9]: $P = \exp(-k_1 t_0)\delta(t_s) + \sqrt{k_1 k_{-1} t_0 / t_s} \cdot I_1(2\sqrt{k_1 k_{-1} t_0 t_s}) \cdot \exp(-k_1 t_0 - k_{-1} t_s)$, where P is the probability density function, t_s is the time from the elution of the original Gaussian part of peak, δ denotes the Dirac delta function and I_1 denotes the Bessel function of imaginary argument. Assumptions: adsorption constant for the slow process $k_1 = 1.67 \cdot 10^{-3}$ s^{-1}, desorption constant $k_{-1} = 1.67 \cdot 10^{-3}$ s^{-1}, capacity factor for the initial Gaussian peak $k'_G = 9.00$, number of theoretical plates $N = 1000$. (A) Hold-up time $t_0 = 60$ s; (B) $t_0 = 120$ s; (C) $t_0 = 300$ s; (D) $t_0 = 600$ s; (E) $t_0 = 1200$ s; (F) $t_0 = 6000$ s.

the stochastic equation, in the lower half of the "peak-transforming mobile-phase residence time region" (Fig. 1B–E), there is "growth" of the tailing portion in the elution peak, formed by solute molecules participating in a slow process during passage through the column. The fractional area of the tailing portion (f_T) increases with mobile-phase residence time (by reducing the mobile phase flow-rate), with a consequent reduction in the leading Gaussian portion of peak formed by solute molecules involved only in a fast process. The fractional area of the Gaussian portion ($f_G = 1 - f_T$) thus decays exponentially with mobile-phase residence time, i.e.,

$$\ln f_G = -k_1 t_0 \quad (1)$$

where k_1 (s^{-1}) is the first-order rate constant of slow adsorption. The hold-up time, t_0 (s), is equal to the residence time of the solute in the mobile phase of the column and is calculated as the elution time of an unretained solute.

When the tailing portion has only a minor fraction of the entire area of the whole peak (Fig. 1B and C), f_T can be evaluated by subtracting, from the whole peak area, the area of the Gaussian function best fitted to the leading edge of the elution peak. This is achieved by the curve-fitting method developed by Ohkuma and Hara [7] with the eight-parameter equation proposed by Cheslar and Cram [10]. This equation is essentially empirical but has been shown to be useful for describing and characterizing tailing peak shapes in conjunction with various chromatographic theories and aspects [11]. The initial estimate determination for the non-linear least-squares procedure involved in our curve-fitting method was modified for obtaining f_T and f_G. The method was described in detail in a previous paper [7].

In an actual LSC system, a non-linear tailing effect may exist even at high solute dilution, probably due to microscopic inhomogeneity of the adsorbent surface. To take this effect into consideration, an asymmetry coefficient, c_N, is introduced into Eq. 1, as the factor by which the fractional area of the Gaussian portion (f_G) must be multiplied to express the true area of the peak portion ($c_N f_G$) which decays exponentially with time t_0. Accordingly, Eq. 1 is modified to

$$\ln(c_N f_G) = -k_1 t_0 \quad (2)$$

where $c_N \geq 1$ since peak tailing is always taken as an excess from the Gaussian function fitted to the leading side of the elution peak.

It should be noted that this asymmetry coefficient is related to the non-linearity of the solute adsorption isotherm and thus c_N depends on variations in local solute concentration and, accordingly, on the degree of peak dispersion which varies with the mobile phase flow-rate. However, for the LSC system used in this study, variation in plate height as determined for the

Gaussian portion of the peak has been shown to be limited within the order of magnitude of the average value over the whole range of flow-rates examined. In particular, in the lower half of the "peak-transforming t_0 region", even the highest value of the relative peak width did not exceed twice the lowest value. c_N may thus be considered as not significantly varying, at least over the lower half of the "peak-transforming t_0 region".

Eq. 2 may be rewritten for rate constant determination using experimental data:

$$\ln f_G = -k_1 t_0 - \ln c_N \quad (3)$$

Because the second term on the right-hand side of Eq. 3 may be assumed to be constant with respect to t_0 within the lower half of the "peak-transforming t_0 region", we can evaluate the apparent rate constant, k_1, from the slope of the experimental $\ln f_G$ vs. t_0 plot.

3.2. Two contributions to mean adsorption time

It is assumed that the delayed solute adsorption process represented by the apparent rate constant, k_1, consists of diffusion and adsorption steps. The inverse of this pseudo-first-order adsorption rate constant ($1/k_1$), i.e., mean adsorption time, is thus divided into the two contributions from diffusion (τ_D) and adsorption (τ_A) steps.

$$1/k_1 = \tau_D + \tau_A \quad (4)$$

where τ_D represents the mean time required for transferring solute molecules from the bulk mobile phase stream to the vicinity of the surface and τ_A the mean time required for replacing solvent molecules adsorbed on the surface by solute molecules (mean adsorption time in a narrow sense).

The contribution of τ_D may be derived primarily from the existence of some kind(s) of pores intervening between the bulk mobile phase stream and stationary phase surface [5,6]. Following the method of describing a pore-diffusional process in porous matrix described by Hage et al. [1], τ_D may be given by the equation

$$\tau_D = \frac{V_m}{k_D V_o} \quad (5)$$

where V_m ($= V_o + V_i$) is the whole volume of the mobile phase contained in the column, V_o is total volume of the mobile phase easily accessible to solute molecules and includes the interstitial volume outside adsorbent particles and a portion of the pore volume inside particles, V_i is total volume of the mobile phase in the pores of adsorbent particles where the slow adsorption process occurs and k_D is the rate constant (s^{-1}) and, although unusual, relevantly describes the "forward" diffusional process in the mobile phase in the direction of solute molecule transfer from the promptly accessible volume V_o to the slowly accessible volume V_i.

It should be noted that the volumes (V_i and V_o) used in this theory are different from those used by Hage et al. [1] for describing the kinetics of a similar "forward" diffusional process of macromolecular solutes transferred from the interstitial volume to the pore volume of adsorbent particles in affinity chromatography. In contrast with the latter case, it is very difficult to specify the spaces or regions which constitute volume V_i—it may be defined as the total volume of "core" regions and/or narrow portions within porous adsorbent particles not promptly penetrated by solute molecules and the volume of such regions or portions should vary with size of the solute molecule.

Let us consider the case of a ternary solvent mobile phase as used in our previous study [7] to improve sample resolution with a silica gel LSC system. This mobile phase consists of non-polar (*n*-hexane) and polar (2-propanol) organic solvent components and water, which was selected to investigate the effect of water on peak tailing. It was found that in this LSC system, peak tailing of most of the samples can be diminished by increasing the water concentration in the mobile phase to 0.5% (v/v). Such a small change in the concentration of one component may have no effect on solute diffusivity (k_D) in the mobile phase contained within a porous matrix. V_i and V_o may not change by altering mobile phase composition. τ_D may thus be considered as con-

stant for a particular solute irrespective of changes in water content, although the values of k_D, V_i and V_o cannot be determined concretely.

However, the composition of the solvent layer adsorbed on the stationary phase surface (adsorbed phase), in which solute–solvent exchange occurs, may change greatly with increase in water concentration in the mobile phase from 0 to 0.5% (v/v). This is because water is most preferentially adsorbed on the silica gel surface. This may also have an effect on the mean time required for solute–solvent exchange (τ_A).

The adsorbed phase formed when a silica gel adsorbent is equilibrated with a mixture of hydrocarbon and aliphatic alcohol solvents, such as *n*-hexane (component 1) and 2-propanol (component 2), is known to consist of a multilayer of alcohol molecules adsorbed on the surface [12]. If a small amount of water (component 3) is added, the alcohol molecules occupying the silica surface (i.e., adsorbed in the first adsorbed layer) may be replaced to a considerable degree by water molecules.

Water is much more strongly adsorbed on the surface of silica gel, and thus desorption of water from the surface may require much more time than any polar organic solvent or solute. The portion of the surface area (surface adsorption site such as a free or bonded silanol group) occupied by a water molecule would not be available for the adsorption of another molecule during such a period of time. From this point of view, we assume that when a water molecule is adsorbed on a surface adsorption site of silica gel, that site is virtually inactivated with respect to solute adsorption. Then, the number of active adsorption sites in unit surface area (the surface density of active sites) decreases with increasing number of water molecules adsorbed on the surface. A decrease in the density of active sites would consequently lessen the chance for a solute molecule, just after reaching the vicinity of surface by diffusion, to collide with an active site. Solute adsorption is thus delayed and mean time for solute adsorption (τ_A) is prolonged.

Because the adsorbed phase formed on the surface consists of components 2 and 3 as discussed above, active adsorption sites [i.e., adsorption sites not adsorbing molecules of water (3)] are only those on which molecules of component 2 are adsorbed. τ_A is, therefore, considered to be inversely proportional to the mole fraction of 2 in the first adsorbed layer, x_{2A}:

$$\tau_A = \frac{1}{k_A x_{2A}} \tag{6}$$

where k_A (s^{-1}) is a constant characteristic of the LSC system (combination of solute, mobile phase and adsorbent), as

$$k_A = k_{A2} N_S A_S \tag{7}$$

where k_{A2} is the second-order rate constant of solute adsorption (solute–solvent exchange) with dimensions of s^{-1} per molarity of active adsorption sites "in the mobile phase" (i.e., in contact with unit volume of mobile phase) (s^{-1} mol^{-1} l). N_S is the total number of moles of solvents adsorbed in the first adsorbed layer per unit surface area of silica gel (i.e., total surface density of adsorption sites) (mol m^{-2}) and A_S is the specific surface area of the silica gel, calculated per unit volume of mobile phase in contact with the surface (m^2 l^{-1}). N_S and A_S for the surface within the "slowly accessible volume" are assumed to be not significantly different from the corresponding values averaged throughout the silica gel column. $N_S A_S$ is the molarity of adsorption sites in contact with unit volume of the mobile phase (mol l^{-1}).

If we rewrite the denominator of the right-hand side of Eq. 6 according to the definition of k_A in Eq. 7, $k_A x_{2A} = k_{A2}$ [the molarity of active adsorption sites in contact with unit volume of the mobile phase]. Eqs. 6 and 7 thus relate the kinetics of solute adsorption (τ_A) with the dynamic equilibrium of competitive adsorption of mobile phase components determining the molarity of active adsorption sites.

By developing the theory for the competitive adsorption equilibrium of mobile phase components, we may relate the mole fraction of component 2, x_{2A}, in the first adsorbed solvent layer, which has been introduced in Eq. 6, to the concentrations of solvent components in the mobile phase, particularly to the water concentration. It is very difficult to treat strictly the

competitive equilibrium of this adsorption system, owing to energetic inhomogeneity of the silica gel surface and multilayer formation of solvents on the surface, influencing the adsorption equilibrium of mobile phase components. Nevertheless, this relation can be expressed provided there is an effective thermodynamic equilibrium between the concentrations (or, more exactly, thermodynamic activities) of solvent components in the mobile phase and first adsorbed layer, as done by Rizzi [13] to investigate a similar LSC system. The expression of x_{2A} obtained by this approach has the form

$$x_{2A} = \frac{x_{2M}\gamma_{2M}}{\sum_{n=1}^{3} K_{n2} x_{nM}\gamma_{nM}} \quad (8)$$

where x_{nM}s and γ_{nM}s ($n = 1, 2, 3$) denote, respectively, mole fractions and activity coefficients of solvent components in the mobile phase. The activity coefficient represents the non-ideality of the component in the mobile phase as a deviation from Raoult's law, i.e., from the state of the ideal condition (reference state) of $x_{nM} = 1$ in the mobile phase. That is, the activity coefficient is the factor by which the actual concentration must be multiplied to determine the effective concentration of the component corresponding exactly to its chemical or physical behaviour (the thermodynamic activity).

The difference between actual and effective concentrations of component n arises mainly from the difference between the free energy of intermolecular interaction in the mobile phase and reference state (pure solvent n). K_{n2}s are the constants of the effective thermodynamic equilibrium of "phase exchange reactions" between solvent components to replace component 2 by component n:

$$n_M + 2_A \rightleftharpoons n_A + 2_M \quad (9)$$

where $n = 1$, 2 or 3, and subscripts M and A indicate that the solvent components are in the mobile phase and first adsorbed layer, respectively. These phase exchange reactions are assumed to be governed by the condition

$$\frac{x_{nA}\gamma_{nA}x_{2M}\gamma_{2M}}{x_{nM}\gamma_{nM}x_{2A}\gamma_{2A}} = K_{n2} \ (n = 1, 2, 3) \quad (10)$$

where x_{nA}s and γ_{nA}s are, respectively, mole fractions and activity coefficients in the first adsorbed layer. The reference state for γ_{nA}s is $x_{nA} = 1$. The other notations are the same as described above. Note that Eq. 8 has been derived from the conditions formulated by Eq. 10, assuming $\gamma_{nA} = 1$. This is because the adsorbed phase on the silica gel surface is known to be nearly ideal, where adsorbed solvent molecules may be localized on to surface silanol groups to undergo such weak interactions with each other as to result in no significant difference in the free energy of interaction due to the difference in the chemical types of interacting molecules.

If we further assume that the hydrocarbon component 1 is hardly capable of competing with polar component 2, which is much more strongly adsorbed on silica gel than non-polar molecules, i.e., $K_{12} \approx 0$, then Eq. 8 reduces to the form

$$x_{2A} = \frac{1}{1 + K_{32}\dfrac{x_{3M}\gamma_{3M}}{x_{2M}\gamma_{2M}}} \quad (11)$$

Eq. 11 shows the mole fraction of active adsorption sites on the surface (x_{2A}) to be a decreasing function of the ratio of mobile-phase activity of water to that of component 2 ($x_{3M}\gamma_{3M}/x_{2M}\gamma_{2M}$). Introducing Eq. 11 into Eq. 6, which is then introduced together with Eq. 5 into Eq. 4, leads to the following equation:

$$\frac{1}{k_1} = \frac{1}{k_D}\frac{V_m}{V_o} + \frac{1}{k_A} + \frac{K_{32}x_{3M}\gamma_{3M}}{k_A x_{2M}\gamma_{2M}} \quad (12)$$

This is the final expression for mean adsorption time of slow solute adsorption process which give rise to peak tailing in LSC. Because the first and second terms on the right-hand side of Eq. 12 are constant, as discussed above, particularly with respect to water concentration in the mobile phase, and K_{32} and k_A are thermodynamic and kinetic constants independent of concentrations of the solvent components, the third term in Eq. 12 predicts that $1/k_1$ should be a linearly increas-

ing function of the ratio of thermodynamic activity of water to that of 2-propanol. The constant terms on the right-hand side of Eq. 12, which represent the $1/k_1$ value at $x_{3M} = 0$, consist of the contributions from diffusion ($V_m/k_D V_o$) and adsorption ($1/k_A$) steps of the slow adsorption process. It should be noted that k_A is the product of the second-order adsorption rate constant (k_{A2}) and molarity of adsorption sites for a delayed chromatographic process, each of which is supposed to be of the same order of magnitude as that of the corresponding quantity for a fast chromatographic process. That is, the second term is not supposed to involve any factors which give rise to differences in the order of magnitude of the kinetics of the slow process in comparison with that of the fast process.

4. Results and discussion

4.1. Elution peak transformation by increasing column hold-up time: growth of the tailing portion of peak

For analysing the transformation of experimental elution profiles in LSC induced by changes in t_0, as expected by the stochastic equation (Fig. 1), chromatograms were recorded with various mobile phase flow-rates in the range 0.01–3.0 ml/min. The sample used was trianisoyldeoxycytidine, as in the previous study [7] as the "tailing solute". The chromatographic data were processed by the curve-fitting method [7], as depicted in Fig. 2, to calculate the fraction f_T of the area of the tailing portion as residual area by subtracting, from the whole peak area, the area under the curve of a Gaussian function which best fitted to the leading edge of the elution peak (Fig. 2B). The results are shown in Fig. 3, in which f_T is plotted over the whole range of mobile phase flow-rates examined. The degree of scattering of f_T data for each flow-rate is indicated in Fig. 3 by the bar representing the range encompassed by the radius of standard deviation from the arithmetic mean of f_T.

It was proved previously [7] that there is no significant contribution from "extra-column effects" to the peak asymmetry (i.e., f_T) for the same LSC system as in the present study, even at the highest flow-rate. This appears to be supported by the finding that an approximately zero f_T value, i.e., an almost ideal Gaussian-form elution profile, could be obtained at the highest flow-rate. The peak asymmetry data in Fig. 3 were thus used to characterize intra-column processes.

It can clearly be seen from Fig. 3 that as the flow-rate is reduced to less than 1.0 ml/min, f_T increases significantly (i.e., the tailing portion

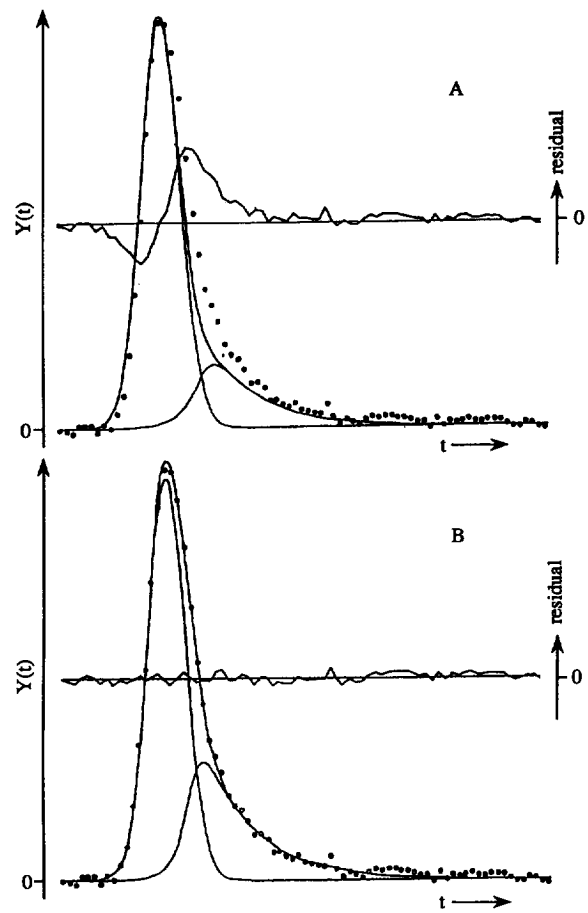

Fig. 2. Least-squares curve fitting for the calculation of the fractional area of the tailing portion of the chromatographic peak obtained from experiment (○), as the departure from the theoretical Gaussian function best fitted to the leading side of the experimental peak. (A) Initial estimate determination process; (B) finally obtained best fit used for the calculation of the fractional area.

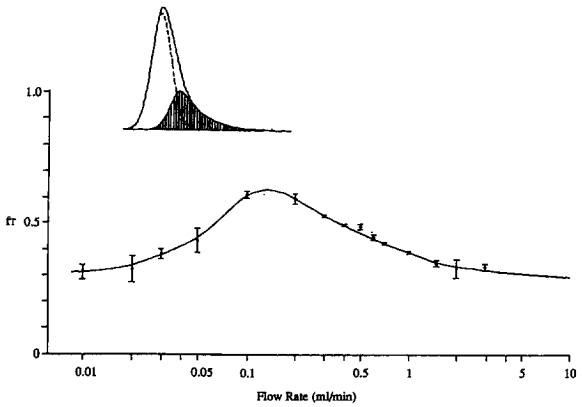

Fig. 3. Variation of the value of fractional area of the tailing portion, f_T, of the chromatographic peak obtained from experiment as a function of the flow-rate of mobile phase. Sample solute, N,3′,5′-trianisoyldeoxycytidine; mobile phase, *n*-hexane–2-propanol–water (80:20:0.3, v/v/v); column packing, LiChrosorb Si 60; column temperature, 28°C.

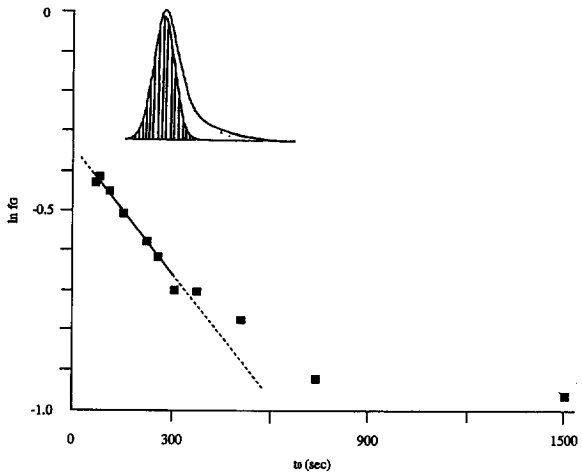

Fig. 4. Application of Eq. 3 to an experimentally obtained ln f_G vs. t_0 plot by the least-squares method to evaluate k_1 and c_N. Sample and chromatographic conditions as in Fig. 3.

grows) from ca. 0.3, calculated as a constant value for the flow-rates higher than 1.0 ml/min, to a maximum of ca. 0.6 near 0.1 ml/min. f_T decreases again to a constant value of ca. 0.3 for flow-rates lower than 0.02 ml/min. Remarkably, this behaviour is consistent with the peak shape change expected on the basis of the stochastic equation assuming a slow adsorption–desorption process along with a normal fast chromatographic process. In this particular instance, the range of flow-rates 1.0–0.1 ml/min (ca. 150–1500 s for t_0) corresponds to the lower half of the "peak-transforming t_0 region", in which the tailing portion of the elution peak grows owing to the slow chromatographic process. The range 0.1–0.02 ml/min (ca. 1500–6800 s for t_0) corresponds to the higher half of the "peak-transforming t_0 region", in which the peak shape returns to a more symmetric original form.

To evaluate the apparent rate constant of the slow adsorption process causing the growth of the tailing portion of the elution peak, the logarithm of the fractional area of the Gaussian portion, $\ln f_G[=\ln(1-f_T)]$, was calculated from the data in Fig. 3 (mean values for f_T at different flow-rates) and plotted over the lower half of the "peak transforming t_0 region", according to Eq. 3 (see Fig. 4). $\ln f_G$ decreases linearly with t_0, particularly in the t_0 range 0–300 s. That is, the fraction of solute molecules that forms the original eluting zone ($c_N f_G$) decays exponentially with t_0 for a first-order process which produces the tailing zone, as described by Eq. 3. The solid linear line in Fig. 4 represents the best least-squares fit of Eq. 3 to the experimental $\ln f_G$ vs. t_0 plot. The reason why the $\ln f_G$ data deviate from a straight line for t_0 higher than 300 s may be explained as follows.

Consider the condition in which $\ln c_N$ is zero (i.e., a non-linear tailing effect does not exist) for simplicity of discussion. It should be recalled that the curve-fitting method in the present study is based on the assumption that in this condition, only the solute molecules which have participated in a slow adsorption–desorption process during passage through the column (called "captured molecules" in our previous paper [7]) can form the tailing portion of the elution peak. Thus, the mole fraction of "captured molecules" can be calculated as the fraction of residual area obtained by subtracting the leading Gaussian area from the whole peak area. As mentioned under Theory and discussed in our previous paper [7], this assumption is valid as long as the "captured molecules" have a minor mole fraction in the total solute molecules injected. That is, f_T may be considered as the mole fraction of

"captured molecules" as long as f_T is small (probably when $f_T < 0.5$). When the fraction of "captured molecules" becomes predominantly large, they occupy most of the peak area not only in the trailing side but also in the leading side of the elution peak. The "uncaptured molecules" thus occupy only a small area of the elution peak, and the Gaussian-shaped distribution of these molecules may not be prominent from the leading profile of the elution peak.

In the latter case, the fractional area of "uncaptured molecules" cannot be decomposed easily and is probably overestimated as f_G in the present curve-fitting method. Therefore, it is natural that the ln f_G vs. t_0 plot in Fig. 4 deviates upwards from the straight line representing the theoretical course of change according to Eq. 3, at t_0 higher than 300 s where the calculated values of f_T exceed 0.5 (ln $f_G < -0.7$). In conclusion, the deviation of the experimental plot from the theoretical straight line would not indicate a mistake in the choice of the theoretical model for describing the kinetics but a consequence of applying the theory beyond the appropriate range of data.

Table 1 lists the values of the rate constant, k_1, and asymmetry coefficient, c_N, obtained by applying Eq. 3 to linear regions of experimental ln f_G vs. t_0 plots at various column temperatures and mobile phase compositions.

4.2. Asymmetry coefficient, c_N

The asymmetry coefficient, c_N, is a measure of peak asymmetry due to non-linear adsorption on the inhomogeneous adsorbent surface at high solute dilution. c_N was evaluated by extrapolating the ln f_G vs. t_0 plot by means of Eq. 3 to the limiting ln f_G value at $t_0 = 0$, corresponding to a flow-rate so high that ln f_G would not be influenced by a slow chromatographic process but not measurable by experiment. The results in Table 1 show that c_N can be decreased to a value approximated to unity by increasing water concentration in the mobile phase to 0.7% (v/v) and simultaneously increasing the column temperature to 70°C. This implies that in this particular condition peak tailing is explained solely by the kinetic effect of the slow chromatographic process as described by the original form of Eq. 1. In fact, Gaussian-form elution profiles can be obtained at the highest flow-rates examined, suggesting an ideal Gaussian asymptotic form of the elution profile in the fast process, just as assumed in the stochastic equation for the elution profile (Fig. 1).

Table 1
Apparent first-order rate constant, k_1, and asymmetry coefficient, c_N, calculated by means of Eq. 3 from data obtained for N,3',5'-trianisoyldeoxycytidine chromatographed on LiChrosorb Si 60

Temperature (°C)	Mobile phase composition *n*-hexane–2-propanol–water (v/v/v)	k_1 (s^{-1})	c_N
28	80:20:0.3	$1.1 \cdot 10^{-3}$	1.4
	80:20:0.4	$9.1 \cdot 10^{-4}$	1.3
	80:20:0.6	$7.3 \cdot 10^{-4}$	1.1
	80:20:0.7	$6.3 \cdot 10^{-4}$	1.1
50	80:20:0.3	$2.3 \cdot 10^{-3}$	1.2
	80:20:0.5	$1.4 \cdot 10^{-3}$	1.1
	80:20:0.7	$1.1 \cdot 10^{-3}$	1.0
70	80:20:0.3	$3.1 \cdot 10^{-3}$	1.1
	80:20:0.5	$2.2 \cdot 10^{-3}$	1.0
	80:20:0.7	$1.5 \cdot 10^{-3}$	1.0

As described under Theory, c_N represents the asymmetry of the distribution of "uncaptured molecules", particularly in terms of the excess area deviating from the Gaussian-form distribution. The distribution of "uncaptured molecules" is formed by the fast chromatographic process and hence the asymmetry of this distribution may be related to the microscopic inhomogeneity of the surface and non-linearity of the adsorption isotherm on the surface. Although the aim of this study was mainly to analyse the apparent rate constant, k_1, the mechanism for the formation of this peak asymmetry is proposed to explain the physical meaning of c_N in Eq. 3 and clarify the reason why c_N can be diminished by increasing the mobile-phase water content and column temperature.

Different types of surface chemical groups of silica gel adsorbents are known to be operative as solute adsorption sites on the stationary phase surface in LSC (free and hydrogen-bonded silanols including geminal and vicinal hydroxyl groups) [14]. We may thus assume that certain adsorption sites of relatively high adsorption energy are present with a small population in comparison with the rest of the (lower-energy) adsorption sites on the stationary phase surface. When the mobile phase in which the solute is dissolved is brought into contact with the adsorbent surface in a LSC column, solute molecules are preferentially adsorbed (localized) on higher energy sites. Because of the small population of higher energy sites on the surface, localized solute adsorption may possibly result in local saturation of higher energy sites, with approximately zero local coverage still maintained for the rest of the adsorption sites, even though the sample solute is highly diluted in the mobile phase.

It is well known that when the surface coverage by solute molecules becomes significant (i.e., increases to a coverage not approximated to zero coverage), the solute distribution coefficient and chromatographic capacity factor decrease significantly compared with those obtained at an approximately zero coverage (as expected from a simple Langmuir-type isotherm model). Such a decrease in the capacity factor at the surface is reflected in an increase in the chromatographic mobility of solute molecules. In the LSC system considered here, the majority of solute molecules move with an average mobility corresponding to a decreased local capacity factor owing to saturation of higher energy sites in contact with the majority of the solute zone. However, a minor fraction of solute molecules may be left behind the majority of the solute zone, moving with lower mobility corresponding to higher local capacity factors reflecting lower local surface coverage with respect to higher energy sites because of the lower solute concentration of the minor zone. This fraction of molecules may be observed as an excess area deviating from the bulk Gaussian-form solute zone formed by the majority of solute molecules (i.e., peak tailing). The ratio of this excess tailing area to bulk Gaussian area may be calculated as an excess of c_N over unity in the present curve-fitting method.

In the framework of this simplified explanation for the formation of peak asymmetry, c_N, the above-described effects of increased water content on c_N are interpreted as follows. When equilibrated with the adsorbent surface, water molecules dissolved in the mobile phase may be adsorbed more preferentially on the higher energy sites than any other solvent or solute molecules. Thus, water molecules may reduce the influence of higher energy sites on solute molecule mobility.

The effect of increased temperature may be due to a reduction in the degree of preference for solute adsorption (localization) on higher energy sites rather than on the rest of the (lower energy) adsorption sites, probably because at high temperatures entropic loss of localized adsorption predominates over its energetic gain.

Asymptotic value of $-\ln f_G$ in the higher half of the "peak transforming t_0 region", estimated from the f_T vs. t_0 plot shown in Fig. 3 in the highest range of t_0($<$0.05 ml/min), represents the asymmetry of asymptotic peak shape approached by increasing the number of delayed adsorption–desorption steps. This value coincides with $\ln c_N$ obtained for the lower half of the "peak trans-

forming t_0 region" (>0.5 ml/min) representing the asymmetry of asymptotic peak shape obtained by the fast chromatographic process. The reason for this coincidence may be that the surface on which the slow chromatographic process occurs is essentially the same, i.e., thermodynamically equivalent with that on which fast chromatographic process occurs.

4.3. Pseudo-first-order rate constant, k_1, of slow adsorption

The adsorption rate constant, k_1, was evaluated from the slope of linear ln f_G vs. t_0 plot, as described above (Fig. 4), by means of the best least-squares fit to Eq. 3; k_1 may thus be the observed rate constant for a pseudo-first-order process of delayed adsorption. The results in Table 1 show that for the sample examined, k_1 is evaluated to be of the order of 10^{-3}–10^{-4} s^{-1} and increases with column temperature and decreases with mobile phase water content. That is, slow adsorption is accelerated by elevating the column temperature and decelerated by increasing the mobile phase water content. The same order of magnitude and behaviour of k_1 were observed for other sample solutes (tribenzoyldeoxycytidine and pyrazine), although not shown in Table 1.

Fig. 5 shows plots of k_1/T vs. $1/T$, presented in a semi-logarithmic coordinate system, at different mobile-phase water concentrations, where T (K) is the thermodynamic temperature. The slope of this type of plot is known to represent the enthalpy of activation for the kinetic process. Increasing the mobile-phase water concentration would not be likely to cause a significant change in the enthalpy of activation for delayed adsorption, suggesting no change in the mechanism of the kinetic process.

4.4. Role of water in the kinetic process of solute adsorption

In Fig. 6, the inverse of the observed adsorption rate constant ($1/k_1$) is plotted against the ratio of the activity of water in the mobile phase to that of the polar organic component of

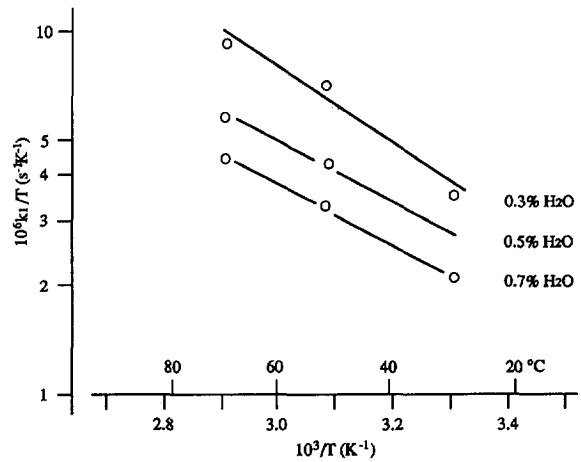

Fig. 5. Plot of k_1/T against $1/T$ to show the dependence of the apparent rate constant of the delayed adsorption process on column temperature. Mobile phase, *n*-hexane–2-propanol (80:20)–water. Sample and column packing as in Fig. 3.

the mobile phase, 2-propanol $(x_{3M}y_{3M}/(x_{2M}\gamma_{2M})$ according to Eq. 12. The mobile-phase activity coefficients of the two components (γ_{2M} and γ_{3M})

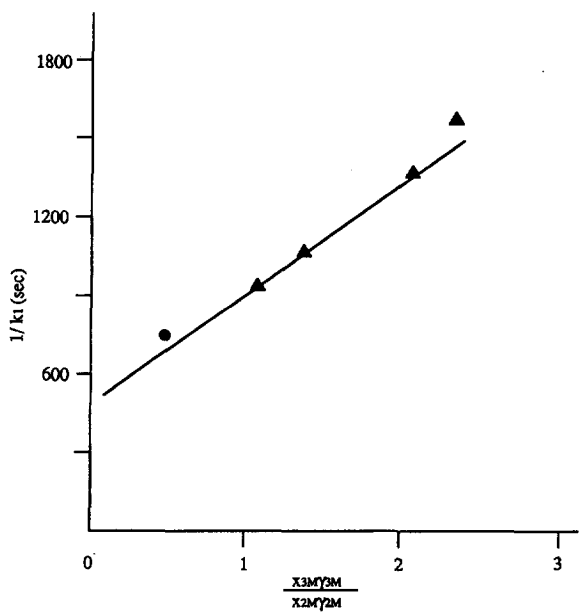

Fig. 6. Representation of the change of the mean adsorption time, $1/k_1$, as a linear function of the ratio of the thermodynamic activity of water in the mobile phase to that of 2-propanol, $x_{3M}\gamma_{3M}/x_{2M}\gamma_{2M}$ by means of Eq. 12. Mobile phase, *n*-hexane–2-propanol [80:20 (▲) or 70:30 (●)]–water. Sample and other conditions as in Fig. 3.

were evaluated for each mobile phase composition by means of the UNIFAC method [15].

The results show that the course of change in $1/k_1$ induced by changes in the mobile-phase concentration of water and/or 2-propanol may be fairly well explained by Eq. 12, which predicts a linear increase in $1/k_1$ with $x_{3M}\gamma_{3M}/x_{2M}\gamma_{2M}$. This supports the fundamental theoretical model assuming that the contribution of solute–solvent exchange on the surface (τ_A) to mean solute adsorption time ($1/k_1$) is, as predicted in Eq. 6, prolonged by decreasing the mole fraction of active adsorption sites on the surface (x_{2A}), which, as in Eq. 11, is a decreasing function of the ratio of the mobile-phase activity of water to that of 2-propanol.

It should be noted in Fig. 6 that the increase in $1/k_1$ observed when decreasing the mobile-phase concentration of 2-propanol from 30% (●) to 20% (▲) with the water concentration kept constant may be attributed not only to decrease in the mobile-phase activity of 2-propanol but also to increase in the mobile-phase activity of water. This is because water exhibits a higher activity coefficient when dissolved in a liquid phase containing a larger amount of hydrophobic non-polar solvent (component 1).

The straight line fitted to the data in Fig. 6 according to Eq. 12 can be extended to indicate that if the mobile-phase activity of water is increased to three times as that of 2-propanol (1:20 in volume, this being the practical limit for miscibility with the *n*-hexane diluent at room temperature), the mean adsorption time ($1/k_1$) will reach ca. 30 min. This implies, in practice, that for a significant fractional area of the tailing portion ($f_T \geq 0.3$, for instance) to be produced by delayed adsorption, a column hold-up time exceeding 10 min is necessary using the LSC system examined. This corresponds to very slow elution with a flow-rate less than 0.2 ml/min. That is, by maximizing the mobile phase water concentration, $1/k_1$ can be prolonged so much as to be of no significance in most instances of LSC under usual elution conditions.

In conclusion, the influence of a small amount of water added to the mobile phase on the slow solute adsorption process causing peak tailing may be explained as being due to inactivation of surface adsorption sites by adsorption of water predominantly competing with the polar organic component of the mobile phase. The predominant adsorption of water may be due not only to the "intrinsic" energetic preference for the adsorption of water on the silica gel surface over that of the polar component (thermodynamic constant K_{32}) but to the high thermodynamic activity of a small amount of water as well, compared with other components comprising the greater part of the rather hydrophobic mobile phase.

4.5. Solute adsorption delayed by diffusion

Eq. 12 may also be used to extrapolate the plot in Fig. 6 to limiting $1/k_1$ at $x_{3M}\gamma_{3M}/x_{2M}\gamma_{2M} = 0$ (i.e., zero water concentration), at which $1/k_1$ cannot be measured owing to certain experimental difficulties discussed previously [7]. The value of this intercept (ca. 500 s) is of the same order of magnitude as $1/k_1$ values (10^2–10^3 s) observed experimentally using water-containing mobile phases as shown in Fig. 6. This indicates that the order of magnitude of $1/k_1$ is determined primarily by the first and second constant terms of Eq. 12.

In the limit of zero water concentration in the LSC system examined, all adsorption sites are active for solute adsorption because they are occupied not by water but by 2-propanol molecules which are capable of being replaced by solute molecules. The second term of Eq. 12 ($1/k_A$) describes the contribution to $1/k_1$ of the solute–solvent exchange process (i.e., adsorption from solution) on an "activated" surface. However, as discussed under Theory, $1/k_A$ cannot be considered to take such a time as to generate the order of magnitude of $1/k_1$ values, because $1/k_A$ is, as in Eq. 7, assumed to be made up by three factors characterizing adsorption kinetics, all of which are considered common to fast and slow chromatographic processes. Indeed, we can tentatively calculate a $1/k_A$ value of smaller than 40 s (i.e., not reaching as high as one tenth of the limiting $1/k_1$ value) from the slope of the plot in Fig. 6, represented by K_{32}/k_A in Eq. 12, assuming

K_{32} to exceed 10, for instance, because of the energetically preferred adsorption of water compared with 2-propanol.

The first term of Eq. 12 ($V_m/k_D V_o$) describes the contribution to $1/k_1$ of the diffusional process in the mobile phase composed mainly of *n*-hexane–2-propanol to transfer solute molecules from the bulk flow stream to the vicinity of the adsorbent surface. This term may probably explain the order of magnitude of the limiting $1/k_1$ value if solute adsorption occurs on a "slowly accessible surface" contained within V_i ($=V_m - V_o$) of the mobile phase which is not promptly accessible to solute molecules because of slow diffusion (low k_D in Eq. 5) in the pores constituting the matrix of the volume V_i.

If we assume that the value of the second term is negligible ($1/k_A \approx 0$), as discussed above, we can estimate the rate constant for the diffusional process that delays solute adsorption. V_i and, thus, V_o in the first term are difficult to determine precisely, but V_i may possibly be as large as $0.1V_m$, because the total area of the surface responsible for the slow adsorption process possibly reaches as much as one tenth that of the rest of the stationary phase surface contained in the column, as will be discussed below. Correspondingly, V_o/V_m may be estimated to be as high as ca. 0.9 and, therefore, k_D may be calculated to be of the order of 10^{-3} s^{-1}. This k_D value is much lower in comparison, for instance, with the order of magnitude of the rate constant of the diffusional process for sample solutes of higher molecular mass, such as immunological proteins (of the order of 10^1 or 10^2 s^{-1}), to be transferred to the surface inside adsorbent particles in affinity chromatography [1] with a porous microparticulate column packing material (Nucleosil 50) similar to that used in the present study (LiChrosorb Si 60). This suggests that the surface responsible for the slow adsorption observed for a sample of low molecular mass examined in the present study may exist deep within the interior of the adsorbent particles of LiChrosorb Si 60, which is not at all (i.e., not geometrically) accessible to macromolecules such as proteins due to the presence of narrow pores. This is consistent with the argument by Hage et al. [1] that protein molecules could probably penetrate only shallow pores of Nucleosil 50.

The above argument by Hage et al. was based on the fact that the rate constant of the pore diffusional process for protein molecules was evaluated to be one or two orders of magnitude higher for Nucleosil 50 than that obtained with a macroporous adsorbent (LiChrospher Si 500). The macroporous adsorbent may be supposed to permit macromolecular samples to access the surface deep within the interior of adsorbent particles. The accessibility of these deep portions of the macroporous adsorbent may result in an increase in the average time required for macromolecules to reach the stationary phase surfaces, in comparison with the case of Nucleosil 50.

For low-molecular-mass samples, however, use of a macroporous adsorbent instead of LiChrosorb Si 60 used in the present study is expected to reduce the time required to reach deep within adsorbent particles that may give rise to peak tailing as observed with a LiChrosorb Si 60 column. Indeed, preliminary experiments with a macroporous adsorbent (LiChrospher Si 1000) showed that the intercept of the plot of $1/k_1$ vs. $x_{3M}\gamma_{3M}/x_{2M}\gamma_{2M}$), as in Fig. 6, was reduced considerably to at least one order of magnitude lower than that obtained with LiChrosorb Si 60, suggesting an increase of the order of magnitude of the rate constant (k_D) of the diffusional process to the deep within the interior of the silica gel adsorbent particles. The precise determination of k_D for LiChrospher Si 1000 by means of the extrapolation of this plot was difficult because $1/k_1$ changed much more steeply with water concentration in the mobile phase, and thus the determination of the intercept was more greatly influenced by scattering of data in comparison with the case of LiChrosorb Si 60. Nevertheless, it may be found that according to Eqs. 7 and 12, the relative steepness of the slope (K_{32}/k_A) of the plot for LiChrospher Si 1000 is interpreted as being due to a higher $1/k_A$ value arising from the lower specific surface area (A_S) of the macroporous adsorbent because the other factors determining K_{32}/k_A are independent of the pore size of the adsorbent. Thus, a higher $1/k_A$ value should be subtracted from the above-

described small intercept $(V_m/(k_D V_o) + 1/k_A)$ for the macroporous adsorbent, LiChrospher Si 1000, to estimate a much smaller contribution of the diffusional process $(V_m/(k_D V_o))$ to the mean adsorption time and a much more than one order of magnitude higher value for k_D in comparison with the case of LiChrosorb Si 60.

4.6. Contribution of slow adsorption to LSC retention at lower flow-rates

It is expected that a slow adsorption–desorption process should contribute to chromatographic retention when t_0 is prolonged up to and beyond the higher half of the "peak transforming t_0 region", where the elution peak profile returns from a tailing shape to a symmetric Gaussian-like form along with increasing t_0. To confirm this contribution, we can find an increase in the solute capacity factor (k'_G) calculated from retention time of the maximum of the bulk Gaussian portion of the elution peak obtained at a flow-rate corresponding to a t_0 in this region with reference to that measured in a lower t_0 region. Fig. 7 shows a typical result of a plot of k'_G vs. t_0 over the whole range of t_0 examined. Obviously, k'_G does not significantly change with t_0 at shorter t_0 but when t_0 is prolonged to more than 4800 s, k'_G increases toward a certain asymptote which may probably lie at a level approximately one tenth higher than the k'_G value measured at a shorter t_0. This increase in k'_G is evidence for the existence of a slow adsorption–desorption process that can contribute to solute retention only at long hold-up times. The ratio of the retention contribution of the slow process to that of the fast process (ca. 1/10) is considered to show the possibility of reflecting the ratio of the total area of the "slowly accessible surface" to that of the easily accessible surface, because the "slowly accessible surface" on which the slow process occurs is supposed to be thermodynamically equivalent to the easily accessible surface on which the fast process occurs, as discussed above.

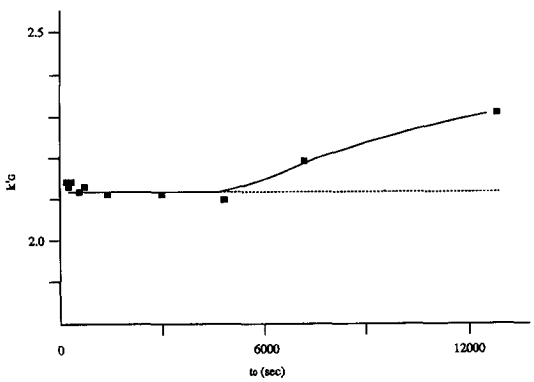

Fig. 7. Plot of the capacity factor, k'_G, calculated from the retention time of the leading Gaussian part of the peak, against hold-up time, t_0. Column temperature, 50°C. Sample and other conditions as in Fig. 3.

5. Conclusion

The kinetics of the slow adsorption process which gives rise to peak tailing in LSC can be analysed from the observation of the growth of the tailing portion of the peak induced by reducing the flow-rate of the mobile phase. The behaviour of the apparent rate constant of the slow adsorption process may be explained by assuming that the adsorption process occurs on the portion of the stationary phase surface existing deep within the interior of the particles of porous microparticulate column packings ("slowly accessible surface") which is thermodynamically equivalent to the rest of stationary phase surface contained in the column and geometrically accessible to solute molecules but require long times to be accessed by solute molecules because of the slow pore diffusional process. The contribution of that portion of stationary phase surface can be observed if the flow-rate is decreased much more so that the elution time is prolonged by more than two orders of magnitude.

Moreover, this paper has demonstrated that the peak tailing phenomenon in the LSC system can be analysed to be composed of the asymmetry caused by the non-linearity of solute adsorption isotherm, which may be incidental to the localized solute adsorption on a small population of high-energy surface sites, as well as of the formation of the tailing zone in the presence

of the slow adsorption process on the "slowly accessible surface". The influence of water added to the mobile phase on both of the mechanisms of peak tailing is through the inactivation of the surface sites by preferential and strong adsorption of water molecules which have a relatively high thermodynamic activity in the hydrophobic environment of the mobile phases used in LSC.

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