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Peculiarities of zone migration and band broadening in gradient reversed-phase high-performance liquid chromatography of proteins with respect to membrane chromatography☆

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ABSTRACT

The peculiarities of zone migration and band broadening in the reversed-phase gradient HPLC of proteins were investigated. In the isocratic mode a critical composition of the mobile phase was found at which all proteins regardless of their molecular mass migrate with equal velocity and have a capacity factor equal to the phase ratio (V_p/V_0), *i.e.*, the same capacity factor as a marker of total accessible volume would have in steric exclusion chromatography. It is shown that steric exclusion conditions are never achieved in gradient HPLC. In the first (adsorption stage) of gradient elution where the separation takes place the velocity of a protein increases until it becomes equal to the velocity of the desorbing solvent front at a critical distance X_0 from column entrance. Strong broadening is characteristic of this stage. In the second (critical) stage the protein travels the remaining distance ($L - X_0$) with the velocity of the solvent. A definition of X_0 is given allowing one very simple calculation of the minimum permissible column length as a function of gradient steepness, mobile phase velocity and protein adsorption parameter. When $x = X_0$ the protein zone has the smallest dispersion. Making $L < X_0$ is especially disadvantageous, as it leads to anomalous bandspreading. The theory of gradient HPLC was refined on this basis and the usefulness of this approach in high-performance membrane chromatography is demonstrated.

INTRODUCTION

In the past 10 years, high-performance liquid adsorption chromatography (HPLAC) of proteins on reversed-phase, ion-exchange, hydrophobic and metal chelate sorbents has been developing rapidly. In all types of HPLAC of proteins, in the first stage protein sorption with a capacity factor $k' > 50$ takes place, followed by the selective desorption of a protein in the concentration gradient of a displacing agent. Protein chromatography in the displacer's gradient takes place according to the "all or nothing" principle, which is manifested in a very sharp dependence of capacity factor on displacer concentration [1,2]. This effect is usually explained by the cooperative interaction between

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protein macromolecules and the stationary phase (multi-site adsorption) [3,4]. With few exceptions, proteins are separated with the aid of gradient elution because isocratic elution is possible only when low-capacity ion-exchange and hydrophobic sorbents are used [5,6]. Such sorbents are not characterized by the above sharp transition from unlimited adsorption to complete desorbtion with a slight increase in the eluting power of the solvent.

An important feature of HPLAC of proteins is that the resolution is independent of the column length or even decreases with increasing column length, which has been reported by several workers [7,8]. This feature makes it possible to use very short columns [9.10] and provides the possibility of chromatography on sorbing membranes: high-performance membrane chromatography (HPMC) [11–15]. In this case sorbing membranes may be considered to be a sorbent layer fixed at the points of particle contact. These membranes, just like the sorbent layer, are characterized by the "interparticle" volume (volume of wide pores through which the flow passes) and the "inner" pore volume (volume of narrow pores filled with stagnant fluid similar to those of perfusion sorbents) [16,17].

The successful development of gradient HPLAC of proteins is based to a considerable extent on the theoretical papers of Regnier and co-workers [4,18] dealing with the mechanism of adsorption chromatography of proteins and on the gradient elution theory developed by Snyder and co-workers [19–26], first for small molecules [19] and later modified for macromolecules [20–26] including proteins [21,22,23]. This theory predicts the retention times and the width of protein peaks and is the basis of process optimization with respect to the degree of separation and the peak capacity [22,24].

However, some important aspects of the elution behaviour have not yet been completely elucidated. First, two problems should be solved. (1) What is the role of steric exclusion in the gradient HPLAC of proteins? In other words, when the eluent composition is programmed, can the zone of desorbed protein migrating along the column acquire the velocity corresponding to the size-exclusion conditions (SEC) of chromatography? (2) Is there a limit to resolution with decreasing column length and, if there is, on what does this limit depend?

In this paper we attempt to answer these questions and to refine on this basis the theory of gradient HPLAC (and, correspondingly, HPMC) of proteins. As in reversed-phase (RP) HPLC the main specific feature of the HPLAC of proteins (sharp transition from adsorption to desorption) is manifested to the greatest extent, this type of chromatography will be considered experimentally.

THEORY

The measure of the affinity of a protein molecule to the stationary phase is the capacity factor k', which is the equilibrium ratio of substance mass in sorbent pores, q_P, and in the adsorption layer, $\bar{q}$, to the substance mass in the mobile phase, q_0:

$$k' = \frac{q_P + \bar{q}}{q_0} = \frac{V_R - V_0}{V_0} \quad (1)$$

where V_R is the elution volume of the protein and V_0 is the elution volume of a standard which is sterically excluded from the pores and does not interact with their surface.

According to the so-called "three-phase" model [27] in which the equilibrium between the free mobile solution in the interparticle volume, the stagnant liquid in pores and the adsorption layer is considered, the capacity factor may be represented as the product of exclusion and adsorption components:

$$k' = k'_{SEC}(1 + k_a) \quad (2)$$

where $k_a = \bar{q}/q_p$ is the adsorption constant,

$$k'_{SEC} = q_p/q_0 = K_{SEC}(V_p/V_0)$$

K_{SEC} is the equilibrium exclusion distribution coefficient (ratio of substance concentration in pores and in the free solution):

$$K_{SEC} = C_p/C_0\,; \quad \kappa = V_p/V_0\,; \quad k'_{SEC} = K_{SEC}\kappa$$

(κ is the ratio of the intraparticle pore volume to the interparticle volume of the column).

It has been shown in papers on the adsorption chromatography of flexible-chain (synthetic) polymers [28–32] on porous sorbents that there is a critical composition of the mobile phase (and an energy of interaction between the segments of macromolecule and the adsorbent surface related to this composition) at which the interphase distribution of all polymers is characterized by the Gibbs energy $\Delta G = -RT \ln K_d = 0$, and the macromolecules regardless of their molecular mass are eluted at $K_d = (C_p + \bar{C})/C_0 = k'/\kappa = 1$, *i.e.*, they have $k' = \kappa$.

At the critical point the exclusion entropy effect is compensated for by the enthalpy effect, *i.e.*, a slight adsorption interaction between the segments of the macromolecule and the inner surface of the adsorbent: $K_{SEC} = 1/(1 + k_a)$, where k_a is the adsorption coefficient.

The critical conditions of protein chromatography may be found (if they exist) by studying the isocratic elution behaviours of several proteins with different molecular masses with a wide range of the mobile phase solvent strength. The phenomenon of critical mobile phase composition and its manifestation in gradient HPLAC of proteins have not been investigated as it is not very evident.

Even the problem of the existence of critical conditions for proteins, *i.e.*, the conditions of chromatography with $k' = k$ has not yet been solved unequivocally. In order to establish the existence of critical conditions, it is necessary to distinguish between the exclusion and the adsorption components of k', *i.e.*, to determine k' from eqn 1 by using only the exclusion volume V_0.

However, some workers [12,20] use other definitions of the capacity factor:

$$k'' = \frac{V_R - V_{SEC}}{V_{SEC}} = \frac{q_p - \bar{q}}{q_p - q_0} = \frac{k'}{1 + \kappa k'_{SEC}} \qquad (3)$$

$$k''' = \frac{V_R - V_m}{V_m} = \frac{k' - \kappa}{1 + \kappa} \qquad (4)$$

where V_m is the elution volume of the substance which is not adsorbed but completely penetrates the intraparticle pores and V_{SEC} is the elution volume of the protein in the absence of adsorption, *i.e.*, under size-exclusion conditions. These definitions do not allow one to distinguish either between macromolecules and small molecules (eqn. 4) or between adsorption and penetration into the inner pores (eqn. 3) and hence all manifestations of the critical phenomenon appear to be masked in experiments and manipulations with capacity factors defined in this manner. Moreover, to detect the critical conditions, monofunctional sorbents should be used, *i.e.*, non-specific adsorption should be completely eliminated.

Because the above requirements have not been probably met, the critical conditions in the HPLAC of proteins have not been found even in investigations dealing specially with this problem [33].

In reversed-phase (RP) ion-exchange (IE) and hydrophobic interaction HPLAC, the dependence of k' on the concentration of the displacing agent may be described according to Kopaciewicz *et al.* [4] by the general equation

$$\log k' = \log k_z - z \log C \qquad (5)$$

where C is the molar concentration of the displacer, *i.e.*, of an organic solvent in RP-HPLAC, or of a salt in IE-HPLAC or of water in hydrophobic interaction HPLAC.

We shall introduce the concept of critical concentration C_c at which $k' = \kappa$, *i.e.*, $K_{SEC}(1 + k_a) = 1$. Then eqn. (5) can be rewritten in the following form:

$$k' = \kappa\left(\frac{C_c}{C}\right)^z \qquad (6)$$

At the high z values (of the order of several tens) which exist in RP-HPLAC the logarithmic dependence of log k' on the concentration passes into a well known linear dependence:

$$\log k' = \log k_w - S\varphi \qquad (7)$$

where φ is the volume fraction of organic solvent or, if the concept of critical concentration is used,

$$\log (k'/\kappa) = S(\varphi_c - \varphi) \quad (8)$$

Retention time in the gradient HPLAC of proteins

In isocratic elution, if eqn. 1 is used the migration velocity of the zone maximum (v) is determined by

$$v = u \cdot \frac{1}{1+k'} \quad (9)$$

where u is the linear elution velocity (with the assumption that the flow passes only through the interparticle volume). Note that the fundamental eqn. 9 is true only for k' defined by eqn. 1, and not eqn. 3 or 4.

Snyder's theory of gradient elution is based on Freiling's equation [34]:

$$\int_0^{V_g^*} \frac{dV}{V_i} = 1 \quad (10)$$

where V_g^* is the reduced retention volume in gradient elution ($V_g^* = V_g - V_m$), V_g is the experimental retention volume, V_m is the total accessible volume of the column and V_i is the instantaneous reduced retention volume.

The solution of eqn. 10 for RP-HPLC under linear gradient conditions is given [21] by the following equation for the retention time:

$$t_R = \frac{1}{SB} \cdot \log [2.3k_0'SB(t_{SEC}/t_0) + 1] + t_{SEC} + t_d \quad (11)$$

at $k'(t)$ defined by the equations

$$k' = k_w \cdot 10^{-S\varphi(t)} \quad (12)$$

and

$$\varphi(t) = \varphi_0 + Bt \quad (13)$$

where φ is the volume fraction of the displacing agent (organic solvent), φ_0 is the initial value of φ at the moment of gradient start, B is the gradient steepness, k_w and S are the parameters of protein adsorption, $k_0' = k_w \cdot 10^{-S\varphi_0}$, t_{SEC} is the time of protein elution under the conditions of size-exclusion chromatography and t_d is the instrumental time lag of a gradient device.

Eqn. 11 makes it possible to attain good coincidence with experimental data [21], although its theoretical basis (Freiling's equation) is not absolutely rigorous as it was derived for a column of infinite length.

If the more correct Drake procedure [35] is used (see Appendix) then we have the following dependence of migration time on migration distance:

$$t(x) = t_0 + \frac{1}{SB} \cdot \log \left[1 + \frac{k_0'}{\kappa}(E-1)\right] \quad (14)$$

and hence the retention time is given as $t_R = t(x = L)$, where $E = 10^{SBx\kappa/u}$, $k_0' = k'$ at $\varphi = \varphi_0$, $t_0 = x/u$ is the migration time of a standard substance that is unable to penetrate into inner pores or to be adsorbed on their surface and L is the column length.

It follows from eqn. 14 that the migration velocity of the chromatographic zone depends on the distance x travelled along the column (at $\kappa << k_0'$) as follows:

$$v(x) = \frac{E-1}{(1+\kappa)E-1} \cdot u = \frac{(1+\kappa)(E-1)}{E(1+\kappa)-1} \cdot u_d \quad (15)$$

where u_d is the displacer's velocity, $u_d = u/(1+\kappa)$, assuming solvent demixing effects to be absent, and u is the mobile phase velocity.

This exponential equation shows that the zone velocity increasing with the distance passed by the zone cannot attain the velocity of the concentration point of the gradient moving at a velocity u_d. However, eqn. 15 enables us to determine the distance X_0 at which the zone velocity $v(x)$ becomes virtually identical with u_d:

$$X_0 = \frac{\lambda u}{SB\kappa} \quad (16)$$

where λ is the parameter characterizing the precision of the fulfillment of the equality $v(X_0) = u_d$. If one inserts X_0 from eqn. 16 instead of x into eqn. 15, it can be seen that $v(X_0) = 0.95u_d$ at $\lambda = 1$ and $v(X_0) = 0.99u_d$ at $\lambda = 2$. These calculations are given for $\kappa = 1$. The theoretical dependence of v on $r = x/X_0$, calculated for $\kappa = 1$ and $\lambda = 1$, is shown in Fig. 1. The value of X_0 plays a fundamental role in the theory of the gradient HPLAC of proteins. It

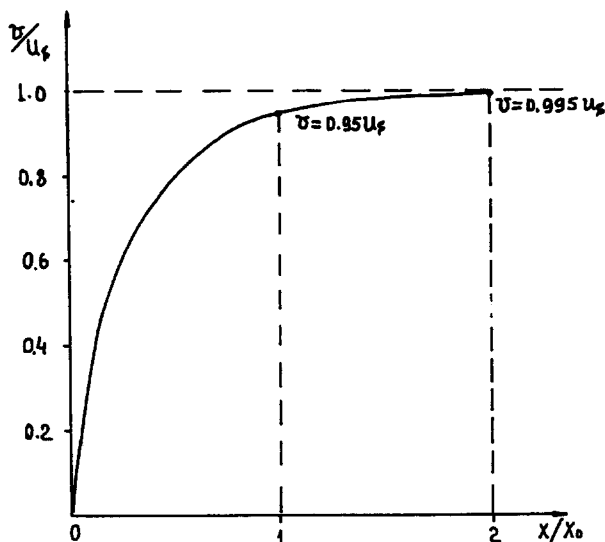

Fig. 1. Ratio of zone velocity (v) to solvent velocity (u_d) as a function of dimensionless migration distance x/X_0 (calculated for $\lambda = 1$ and $\kappa = 1$).

will be shown further that X_0 means the critical distance along the column at which the protein zone comes to the end of its movement in the adsorption mode of chromatography and starts migration according to the laws of critical chromatography.

Eqns. 9 and 15 may be used to obtain the following dependence of the instantaneous capacity factor in gradient elution on the distance x passed along the column:

$$k'(x) = \frac{\kappa E(x)}{E(x) - 1} \quad (\text{at } k' >> \kappa) \tag{17}$$

where $E(x) = 10^{x/X_0}$ at $\lambda = 1$.

It should be noted that Snyder's theory gives the following expression for the instantaneous capacity factor:

$$k'(x) = \frac{1}{2.3SBt_m}$$

or in our designations and taking into account that $t_m = L/u_d$:

$$\tilde{k}'(x) = \frac{\kappa}{2.3(x/X_0)(1 + \kappa)} \tag{18}$$

Expanding eqn. 17 into a series and taking only the first two terms, which is possible only at $x/X_0 << 1$, we can obtain a similar but not identical equation:

$$\tilde{k}'(x) = \frac{\kappa}{2.3(x/X_0)}$$

This means that Snyder's theory of instantaneous capacity factor should work well only for $x << X_0$, *i.e.*, either for short columns or only for the upper part of a long column or when S is small, *i.e.*, for small molecules. However, even in this case the definition of k' by eqn. 4 instead of eqn. 1 leads to the wrong results near the critical point ($k' = \kappa$).

At a column length $L > X_0$ the elution time of the protein zone is given in our theory as $t_R = t(x = X_0) + (L - X_0)/u_d$ and hence from eqns. 8, 14 and 16 it follows that

$$t_R = t(\varphi_c) + t_d + \frac{L(1 + \kappa)}{u} \tag{19}$$

where $t(\varphi_c)$ is the time of the gradient to generate the critical concentration:

$$t(\varphi_c) = (\varphi_c - \varphi_0)/B$$

and t_d is the delay time of a gradient device.

Zone spreading in the gradient HPLAC of proteins

Now the relationships of zone spreading in the gradient HPLAC of proteins will be considered. This problem has been investigated in a series of papers by Snyder's group [19,22,23,25]. As the theoretical model developed by these workers has been repeatedly improved, we shall deal with the latest variant [23,25].

The following expression is used for peak standard deviation (in time units):

$$\sigma_t = 0.5J(1 + \tilde{k}')t_m\left[\frac{H(k')}{L}\right]^{1/2} \tag{20}$$

where H is the height equivalent to a theoretical plate (HETP) and $\tilde{k}'$ is the instantaneous value of k' in the middle of the column:

$$k' = \frac{1}{1.15SBt_m} = \frac{1}{1.15b} \tag{21}$$

$t_m = L/u_d$ is the time of the displacer's movement along the column, $H(\tilde{k}')$ is the value of HETP corresponding to $k' = \tilde{k}'$ and J is the empirical factor of additional spreading:

$$J = 0.99 + 1.7b - 1.35b^2 + 0.48b^3 - 0.06b^4 \quad (22)$$

In this model the dependence of HETP on velocity is given by Knox's equation [36]:

$$h = \frac{H}{d_p} = \tilde{A}v^{1/3} + \tilde{B}/v + \tilde{C}v \quad (23)$$

where $v = ud_p/D_m$ is the reduced velocity. It is assumed that the coefficient $\tilde{A}$ does not depend on k' and the dependence of the coefficients $\tilde{B}$ and $\tilde{C}$ on $\tilde{k}'$ is given by

$$\tilde{B} = 1.1 + b'\tilde{k}' \quad (24)$$

and

$$\tilde{C} = \frac{1 - \tilde{x} + \tilde{k}'}{(1 + \tilde{k}')^2 \cdot 15(B - 1.2b'\tilde{x})}\rho \quad (25)$$

where b' is the parameter of surface diffusion ($0 < b' < 0.25$), $\tilde{x} = 1/(1 + \kappa)$ and $\rho = D_p/D_m$ is the ratio of diffusion coefficient in pores and in the free solution. The ρ value is a decreasing function of r_{sp} (ratio of the Stokes protein radius R to the pore radius r) and can be found from the equation

$$\rho = 1 - 1.83r_{sp} + 1.21r_{sp}^3 - 0.38r_{sp}^5 \quad (26)$$

Snyder's model predicts with high precision the width of the protein peak in gradient HPLAC. Thus, the average value of R_w (ratio of experimental to calculated peak width) is in the range $0.83 < R_w < 1.3$, and relative standard deviation of σ_t is 10–33%. For systems that are not complicated by conformational transitions, aggregation, etc. ("well behaved systems") the protein width may be calculated with a relative standard deviation less than 17%. However, when Snyder's model is used, it is necessary to correct the calculated value of σ_t with the aid of an empirical factor J (eqn. 22), the physical meaning of which, unfortunately, has not been elucidated. Moreover, the experimental dependence of HETP on k' and u (see Fig. 3) found by us does not agree with Snyder's model.

We shall determine zone spreading in the gradient chromatography of proteins taking into account both the chromatographic spreading and the gradient contraction of the protein zone. As the migration velocity of the zone changes continuously, the local HETP (HETP at a distance along the column x) depends on the distance passed by the zone. At the column end, the protein zone acquires the velocity $v(x = L)$ and its time dispersion is determined by the equation

$$\sigma_t^2 = \frac{\sigma_L^2(x = L)}{v^2(x = L)} = \frac{\int_0^L H(x)\,dx}{v^2(x = L)} \quad (27)$$

where $\sigma_L(x = L)$ is the dispersion at the point $x = L$ in length units.

As a result of the concentration gradient of the displacer along the column, the protein zone is subjected to gradient contraction, because the molecules that have diffused to the front boundary of the zone where $\varphi < \varphi_c$ acquire the velocity $v(x + \Delta x)$ and those that have diffused to the rear boundary where $\varphi < \varphi_c$ acquire the velocity $v(x - \Delta x)$. These velocities are lower and higher, respectively, than the velocity of the zone centroid $v(x)$.

The dynamics of the spreading and gradient sharpening of zones may be described by the following differential equation (its derivation is given in the Appendix):

$$H(x) = H_0(x) - \left[4.6\kappa(1 + \kappa)\frac{SB}{u} \cdot \frac{E(x)}{E(x)(1 + \kappa) - 1}\right]\sigma_L^2 \quad (28)$$

To solve eqn. 28, the function $H_0(x)$ should be known. It is determined by the dependence of $H(k')$ on the distance passed along the column through the dependence of the instantaneous capacity factor $k'(x)$ on this distance (eqn. 17).

The general equation for HETP may be written in the following form [37]:

$$H = A^*u + B^*/u + \sum C^*u \quad (29)$$

The character of the dependence of the coefficients A^*, B^* and C^* on k' cannot be considered to be precisely determined because in this case there are theoretical disagreements [37–40], and the scarcity of experimental data does not make it possible to prefer one of these approaches. Moreover, the function $H(k')$ is different for different processes limiting the mass transport velocity: diffusion in the mobile zone, diffusion in the stationary phase (in the immobile zone) and the sorption–desorption reaction.

In the Results and Discussion section we shall show that in RP-HPLC of proteins at linear elution velocities $u > 0.5$ mm/s the first two terms in eqn. 29 related to flow anisotropy (A^*) and molecular diffusion (B^*) may be neglected.

EXPERIMENTAL

Instrumentation

A Kh-Zh-1311 microcolumn gradient chromatograph was used for the chromatography of proteins. It includes two syringe pumps with a variable flow-rate of 1–200 μl/min and with a syringe volume of 1.5 ml and a fluorimetric detector with a 0.3-μl cell and a 0.5-μl injection valve. Proteins were detected by measuring the fluorescence of tryptophan with excitation at 220 nm and collection of the emitted fluorescence radiation at 320 nm. In some instances detection was carried out with the aid of a Shimadzu SPD2 AM spectrophotometric detector at 280 nm.

Columns

The RP-HPLC of proteins was carried out on PTFE microcolumns (30–200 nm × 0.5 mm I.D.) packed with Nucleosil 300-C_4 and 500-C_4 (Macherey–Nagel) or with MPS-300-C_4 alkylated macroporous glass (LenChrom). The last sorbent was prepared by Unger *et al.*'s procedure [41] by treating chlorinated macroporous glass with butyl lithium. The columns were packed at a pressure of 12 MPa from a 20% slurry in carbon tetrachloride with subsequent pumping (without pressure release) of 50% aqueous methanol.

Reagents

Water–acetonitrile systems (LiChrosolv, Merck) with the addition of 0.1–0.3% of trifluoroacetic acid (TFA) (Pierce) were used as the mobile phase.

The proteins ribonuclease A, bovine serum albumin (BSA), thyroglobulin, α-chymotrypsinogen A, immunoglobulin A, conalbumin, lactalbumin and γ-globulin were purchased from Serva and the bacterial ribonuclease M from Reakhim.

As a marker of interparticle volume V_0 in size-exclusion chromatography thyroglobulin was used ($M_r = 660\,000$) and as marker of the total accessible volume $V_T = V_0 + V_P$ *p*-aminobenzoic acid (Reakhim) was used with elution with 70% acetonitrile.

RESULTS AND DISCUSSION

The experimental dependence of k' on the composition of the mobile phase obtained in a series of isocratic experiments for several proteins is shown in Fig. 2. At $k' >> 1$, log k' changes linearly with displacer concentration, *i.e.*, in accordance with eqn. 7. In the isocratic mode in the region of $k' = \kappa$, transition from adsorption to exclusion chromatography occurs. Just as for flexible chain homopolymers [28–32], there is a critical composition at which all proteins regardless of their molecular mass migrate with $k'/\kappa = 1$. It can be seen from Fig. 2 that the $k'(\varphi)$ dependences for the four proteins under investigation intersect at one point which corresponds to the critical composition of the eluent $\varphi_c = 0.425$.

The only exception is chymotrypsinogen A (curve 5), for which $\varphi_c = 0.46$. As proteins are copolymers of 20 different amino acids, are in a globular form and the globular–random coil transition does not take place in HPLAC, the detection of the identical critical composition of the eluent φ_c for a number of proteins is unexpected. The decrease in k' in the range of $\varphi > \varphi_c$ (*i.e.*, in the size-exclusion mode) is probably determined by the increase in the effective size of the protein molecule with a change in

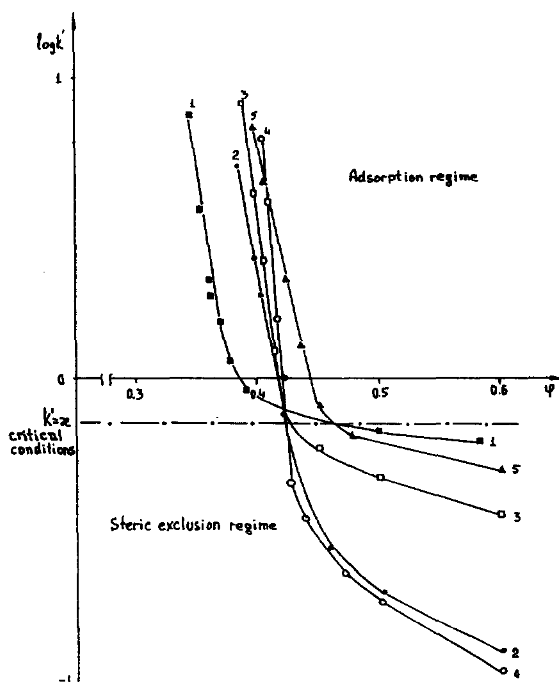

Fig. 2. Logarithmic plot of capacity factor, k', as a function of volume fraction of acetonitrile in the mobile phase, φ. The data were obtained in isocratic experiments for the following proteins: (1) ribonuclease M ($M_r = 12\,200$), (2) bovine serum albumin (66 500), (3) lactalbumin (17 400), (4) conalbumin (80 000) and (5) chymotrypsinogen A (24 000). Column 150 × 0.5 mm I.D., packed with Nucleosil 300-C_4 ($x = \kappa = 0.75$).

eluent composition (it expands as the solvent becomes less polar).

In order to check the conclusion that the velocity of the displacer front is the lower limit of the zone migration velocity in gradient elution, experiments were carried out on the stepwise desorption by 70% acetonitrile ($\varphi > \varphi_c$) of proteins previously adsorbed at $\varphi_0 = 0$. The results are given in Table I. It can be seen that in stepwise desorption all proteins migrate at the velocity of the front of the desorbing solvent u_d, whereas under isocratic elution conditions (φ = 0.7) a size-exclusion effect is distinctly observed (decrease in t_R with increasing molecular mass of protein). These data indicate that under gradient conditions k' cannot (in contrast to Stadalius *et al.*'s opinion [21]) decrease to $k' = k'_{SEC}$ even with a very steep gradient, which means that steric effects do not influence protein separation carried out with the aid of gradient RP-HPLC.

The results presented in Figs. 1 and 2 and Table 1 show that two regions of changes in the composition of the mobile phase in the gradient RP-HPLC of proteins may be distinguished: the precritical region $\varphi \leqslant \varphi_c$, $x \leqslant X_0$ in which the protein zone migration obeys eqn. 15 and the critical region $\varphi \geqslant \varphi_c$, $x \geqslant X_0$ in which the protein migrates at a virtually constant velocity equal to that of the displacer front $v \approx u_d$. It is important that the velocity of motion of all proteins in the

TABLE I

RETENTION TIMES OF PROTEINS ON A REVERSED-PHASE SORBENT IN STEPWISE DESORPTION AND IN ISOCRATIC ELUTION MODES OF HPLC

Compound	Molecular mass	t_R (min)	
		Stepwise desorption, 10–70% CH_3CN	Isocratic elution, >70% CH_3CN
Ribonuclease A	19 700	0.76	0.66
α-Chymotrypsinogen A	24 000	0.76	0.62
BSA	66 500	0.74	0.54
γ-Globulin	169 000	0.75	0.52
Immunoglobulin A	400 000	0.74	0.50
Sodium azide	65	–	0.76
Solvent front	–	0.73	–

critical region is the same. Hence no additional zone separation takes place here. However, the additional band spreading occurs as a result of the further increase in the number of random walk steps, *i.e.*, the number of acts of sorption–desorption.

We shall now define the main differences between the model of protein retention proposed here and Snyder's theory. Freiling's equation (eqn. 10) on which Snyder's model is based is a particular case of eqn. A4 obtained on the assumption that the velocity of the chromatographic zone is much lower than that of the displacer u_d. Hence, eqns. 11, 18 and 21 derived by Snyder for the retention time of the protein and instantaneous capacity factor also refer to a particular case of eqns. 14 and 17 and should be rigorously valid only at relatively low values of $r = x/X_0$. They may be obtained from eqns. 14 and 17 by expanding the functions into series taking into account the fact that k' in Snyder's model is not determined from eqn. 1 but as $k'' = (t - t_{SEC})/t_{SEC}$.

For checking eqn. 14, which determines the retention time of the protein in gradient RP-HPLC, the values of k_w and S were obtained for a number of proteins from isocratic experimental data (Table II). These values are compared in Table II with the values of k'_w and S calculated from eqn. 14 by the method of successive approximations according to the results of two gradient experiments with the variation in φ_0, B and u. Good agreement between the experimental and calculated data indicates that eqn. 14 is valid.

We shall now consider the experimental data on peak spreading in the RP-HPLC of proteins. The dependence of the isocratic HETP on k' for a number of proteins on the variation in the composition of the mobile phase is shown in Fig. 3. It is clear that in the precritical region ($k' > \kappa \approx 0.8$) HETP increases drastically with increase in k'. The higher the molecular mass (M_r) of the protein, the greater is this increase although the problem of plate height dependence on M_r needs further investigation. These experimental data were found to be consistent with the following dependence of HETP on u and k':

$$H_0 = A^* + \omega u + \alpha u[k'/(1 + k')]^2 \quad (30)$$

To determine the velocity dependences of HETP terms at different k' values, the dependence of H/u on $[k'/(1 + k')]^2$ was investigated. It was found that at $u > 0.5$ mm/s the HETP depends linearly on u. Moreover, the value of A^* (0.03 mm $< A^* <$ 0.06 mm) is small and may be neglected, just as the term related to molecular diffusion.

Fig. 4 shows the dependence of H/u on $[k'/(1 + k')]^2$ for (1) ribonuclease M and (2) conalbumin. It can be seen that this dependence is linear and the results obtained at different elution velocities fall on this line with sufficient precision.

The theoretical basis of the dependence $H(k')$

TABLE II

PARAMETERS OF PROTEIN ADSORPTION (LOG k_w AND S) OBTAINED IN ISOCRATIC EXPERIMENTS OR CALCULATED FROM PARALLEL GRADIENT RUNS

Column, 150 mm × 0.5 mm I.D. (Nucleosil 300-C_4); mobile phase, water–acetonitrile containing 0.1% TFA; acetonitrile content varied.

Protein	Log k_w		S	
	Isocratic	Gradient	Isocratic	Gradient
Ribonuclease M	14.2	14.3	38.0	38.1
Lactalbumin	10.1	10.0	23.9	24.0
Conalbumin	27.0	27.5	65.1	64.8

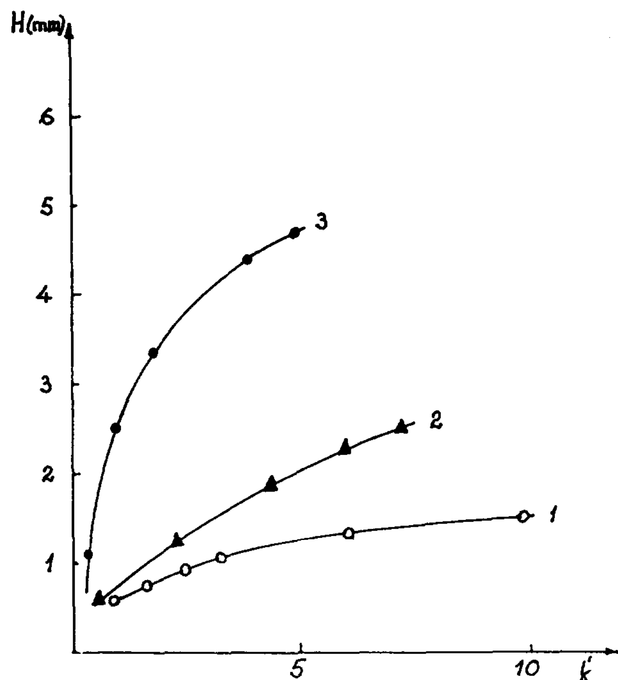

Fig. 3. Experimental dependence of isocratic HETP on capacity factor, k', for the proteins (1) ribonuclease M (M_r = 12 200), (2) lactalbumin (17 400) and (3) conalbumin (80 000).

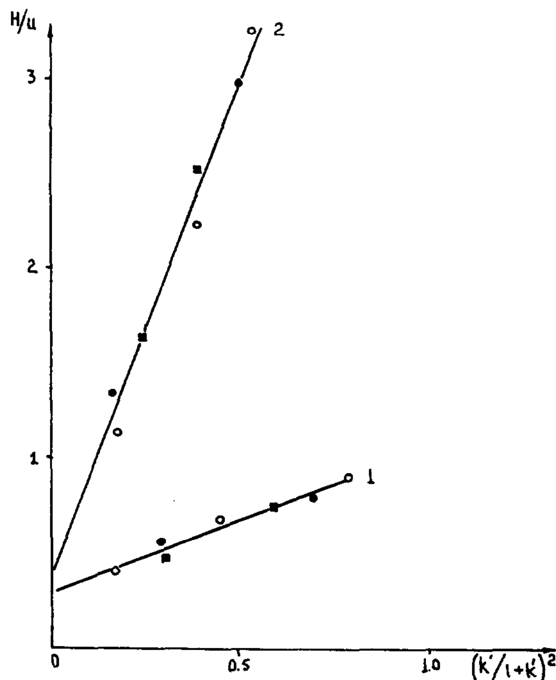

Fig. 4. Ratio of experimental isocratic HETP to linear elution velocity (u) as a function of $[k'/(k'+1)]^2$ for the proteins (1) ribonuclease M and (2) conalbumin. Flow-rate: ○ = 10; ● = 20; ■ = 30 μl/min. H/u in s.

stated above and given by eqn. 30 can be found in Giddings' book [42]. In this book the term independent of k' is related to external diffusion mass transport: $\omega = \tilde{\omega} d_p^2/D_m$ and the term proportional to $[k'/(1+k')]^2$ is determined by internal diffusion resistance to mass transport (when adsorption exists) according to eqn. 4.6–44 in ref. 41. This equation may be written by using our designations and a three-phase adsorption model (eqn. 2) as follows:

$$\alpha = \frac{1}{30} \cdot \frac{d_p^2}{D_s} \cdot \frac{k'(1+k_a)}{(1+k')^2} = \frac{1}{30} \cdot \frac{d_p^2}{D_s K_{SEC} \kappa} \left(\frac{k'}{1+k'}\right)^2 \quad (31)$$

where D_s is the diffusion coefficient in the stationary phase, D_m is the diffusion coefficient in the mobile phase and d_p is particle diameter of the sorbent.

The isocratic data shown in Fig. 4 were used to determine the values $\alpha_1 = 0.52$ s and $\omega_1 = 0.4$ s for ribonuclease M and $\alpha_2 = 2.4$ s and $\omega_2 = 0.3$ s for conalbumin.

The calculated dependences σ_t^2/t_0 for these two proteins in gradient elution were obtained by using the above values of α and w and are shown in Fig. 5 as a function of the dimensionless parameter $r = x/X_0$ (solid lines). Fig. 5 also shows the experimental points for gradient conditions of chromatography obtained by the variation in $r = x/X_0$ with the aid of changes in x, B and u. The calculated and experimental values are in relatively good agreement: the average value of $R_W = \sigma_t(\exp)/\sigma_t(\text{calc})$ is 1.05 for conalbumin and 1.1 for ribonuclease M, and relative standard deviations of the calculated σ_t are 11 and 15%, respectively. It should be noted that a relatively wide range of r was investigated: $0.1 < r < 3.1$. The fact that the values of σ_t^2/t_0 obtained at different velocities fall on the same curve is additional evidence for the conclusion that the contribution of the terms A^* and B^*/u to the HETP value is negligible.

The type of the dependence of protein zone

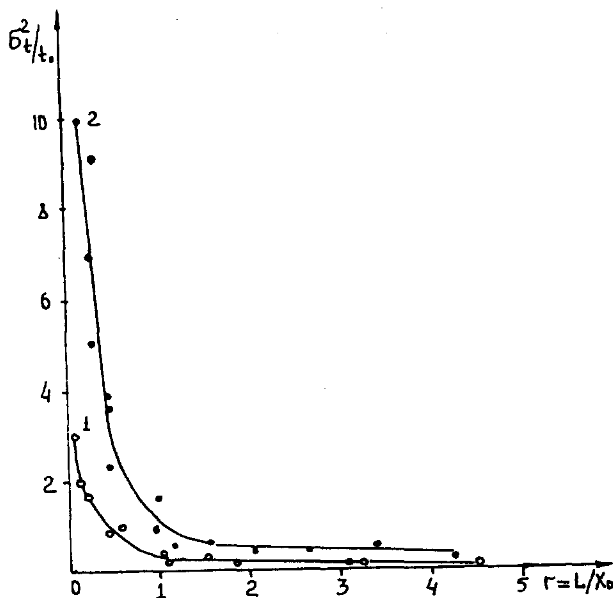

Fig. 5. Ratio of peak variance (σ_t^2) to the time of passage of the mobile phase through the interparticle voids of the column (t_0) as a function of dimensionless column length (L/X_0) in gradient HPLC of proteins. Theoretical dependences were calculated by means of eqn. A17. Experimental points were obtained in gradient runs with varying column length, from rate and gradient steepness. σ_t^2 in s.

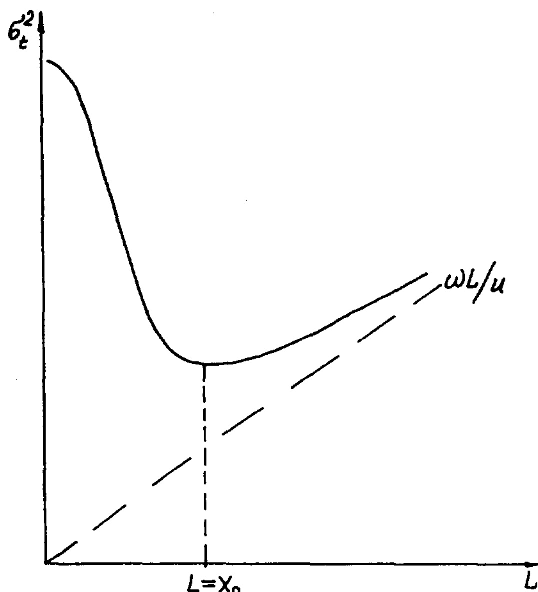

Fig. 6. Graphical representation of the character of the dependence of peak variance on column length in gradient RP-HPLC.

dispersion on column length is shown in Fig. 6. In the precritical region ($L << X_0$) the zone is hardly broadened, as it is eluted with $k' >> \kappa$. At the point $L = X_0$ the variance of the zone is a minimum. At $L > X_0$ there is no gradient compression and the variance becomes proportional to the square root of the column length.

CONCLUSIONS

The relationships considered here are characteristic of columns packed with the usual hydraulically impermeable sorbents for HPLC, which were used in this work. If we pass to perfusion chromatography [16,17,41], in which the eluent moves both in the interparticle volume and in gigantic (giga-dimensions) sorbent pores, it is characterized by the situation in which zone spreading at high elution velocities no longer depends on the eluent velocity, as has been shown elsewhere [43,44]. HPMC on polyglycidyl methacrylate (PGMA–EDM) membranes [11–16] is probably characterized by just this situation because in HPMC [13] the zone width does not change over a relatively wide range of elution velocities. Hence the main features of the HPMC of proteins on the PGMA–EDM membranes include the relationships for the critical chromatography of proteins developed in this paper and the spreading relationships characteristic of perfusion chromatography.

The concept of the critical distance X_0 (eqn. 16) discussed in this paper is of fundamental importance to membrane chromatography. For two proteins with adsorption parameters S_1 and S_2 ($S_2 > S_1$) and corresponding to these parameters critical distances $X_{0,1}$ and $X_{0,2}$ ($X_{0,2} < X_{0,1}$), the distance $X_{0,1}$ will correspond to the optimum thickness of the adsorption layer ($L_{opt} = X_{0,1}$).

At this distance one would obtain the highest separation between peak maxima and the smallest dispersion of peak 1 with peak 2 being slightly broader than it would be at the distance $X_{0,2}$. However, in the case when $L > X_{0,1} > X_{0,2}$ the elution behaviour is much worse, as there is no additional separation, but both peaks become broader. The situation should be extremely bad when $L < X_{0,2} < X_{0,1}$, as in this case the separa-

tion is not at its maximum, but the peaks should be strongly broadened.

Although the column length or membrane thickness may be calculated by means of commercially available software, based on Snyder's theory (Drylab 6), the use of the parameter X_0 proposed in this paper makes all calculations and evaluations extremely simple.

SYMBOLS

A^* contribution of flow anisotropy to the plate height. In general (eqn. 29) A^* is a function of the linear elution velocity (u). At high flow-rates A^* becomes independent of u and is given as $A^* = 2\lambda d\rho$, where λ is the dimensionless coefficient of eddy diffusion and $d\rho$ is the particle size

B steepness of linear gradient (eqn. 12)

B^* coefficient taking into account molecular diffusion in eqn. 29 which describes the dependence of the plate height on elution velocity (u)

b dimensionless parameter of Snyder's theory (eqn. 21); $b = SBt_m$

b' parameter of surface diffusion (eqn. 25)

C molar concentration of the displacing agent (eqn. 5)

C_c critical concentration of the displacing agent at which $K_{SEC}(1 + k_a) = 1$ (eqns. 6 and 8)

C^* coefficient taking into account intraparticle diffusion and adsorption in eqn. 29 which describes the dependence of the plate height on elution velocity (u)

D_m diffusion coefficient in the mobile phase

D_s diffusion coefficient in the stationary phase (eqn. 31)

d_p particle diameter of the packing material

E dimensionless function of x/X_0 (eqns. 14 and 15); $E = 10^{SBx\kappa/u} = 10^{x/X_0}$ ($\lambda = 1$)

F flow-rate (volumetric)

H plate height (height equivalent to a theoretical plate, HETP)

H_0 plate height in the absence of gradient contraction (eqn. A10)

k' capacity factor (dimensionless) (eqn. 1)

k_a adsorption coefficient (dimensionless) (eqn. 2)

k'_{SEC} steric exclusion capacity factor (eqn. 2)

K_d interface distribution coefficient; $K_d = k'/\kappa$

k'', k''' incorrect definitions of the capacity factor (eqns. 3 and 4)

k_W capacity factor (in RP-HPLC) in water mobile phase (organic solvent is absent)

k'_0 capacity factor (in RP-HPLC) in the starting eluent in gradient elution ($\varphi = \varphi_0$)

J Snyder's empirical factor of band broadening (eqn. 22)

L column length

r dimensionless distance; $r = L/X_0$ (eqn. A17)

S dimensionless protein adsorption parameter (in RP-HPLC) (eqns. 7 and 8)

t time

t_R retention time

t_{SEC} retention time in steric exclusion conditions

t_d delay time of the gradient device

t_0 time of mobile phase passage through interpartical voids; $t_0 = x/u = V_0/F$

t_m retention time of a substance which does not interact with the sorbent, but penetrates into its pores

u linear elution velocity; $u = xF/V_0$

u_d solvent velocity; $u_d = u/(1 + \kappa)$, assuming solvent (displacer) demixing effects are absent

V_R retention volume

V_0 interparticle void volume of the column

V_p inner pore volume of the sorbent

V_{SEC} retention volume in steric exclusion conditions

v migration velocity of the zone maximum (eqn. 9)

x distance along the column

X_0 fundamental parameter of critical distance (definition given by eqn. 16)

α auxiliary parameter, taking into account intraparticle diffusion (time units) (eqns. 30 and 31)

λ auxiliary parameter in eqn. 16; $\lambda = 1$

κ phase ratio; $\kappa = V_p/V_0$

ω auxiliary parameter (time units) in eqn. 30; $\omega = \tilde{\omega} d_p^2 / D_m$
σ standard deviation
σ_L peak standard deviation in length units
σ_t peak standard deviation in time units
τ auxiliary time variable (eqn. A2)

APPENDIX

If the velocity of zone migration is given as a function of the displacer concentration $v = f(\varphi)u$ and the gradient function $\varphi(t)$ is known, then it is possible to write a differential equation of zone migration in the following form:

$$\frac{dx}{dt} = uf\left[\varphi\left(t - \frac{x}{u_d}\right)\right] \tag{A1}$$

where u_d is the displacer velocity, $u_d = u/(1 + \kappa)$.

The integration of eqn. A1 enables us to obtain the distance x passed by the zone as a function of time t. With this aim a new variable was introduced:

$$\tau = t - x/u_d \tag{A2}$$

in which the additional time x/u_d has been taken into account. This time is necessary for a concentration point of the gradient moving at a constant velocity $u_d = u/(1 + \kappa)$ to overtake the sample which has passed the distance x. Then eqn. A1 becomes

$$u_d\left(1 + \frac{d\tau}{dt}\right) = uf[\varphi(\tau)] \tag{A3}$$

The solution of this equation:

$$t = \int_0^{\tau} \frac{d\tau}{1 + u/u_d \cdot [\varphi(\tau)]} = F(t) \tag{A4}$$

gives the dependence of the distance x passed by the zone on time t:

$$x = u_d[t - F^{-1}(\tau)] \tag{A5}$$

where $F^{-1}(\tau)$ is the inverse function of $F(\tau)$.

It follows from eqns. 7 and 12 that in RP-HPLC

$$f(\varphi) = \frac{1}{1 + k_w 10^{-S\varphi}} \tag{A6}$$

In this case eqn. A5 has for the linear gradient $\varphi(t) = \varphi_0 + Bt$ the following solution:

$$t(x) = \frac{x}{u} + \frac{1}{SB} \cdot \log\left[1 + \frac{k_0}{\kappa}(E - 1)\right] \tag{A7}$$

where $E = 10^{-SBx\kappa/u}$, $k_0 = k_w 10^{-S\varphi_0}$.

The migration velocity of the chromatographic zone depends on the distance x passed along the column as follows:

$$v(x) = u_d\left[1 - \frac{\frac{\kappa}{(1+\kappa)}\left(1 - \frac{\kappa}{k_0}\right)}{E - \frac{1}{(1+\kappa)}\left(1 - \frac{\kappa}{k_0}\right)}\right] \tag{A8}$$

As $\kappa << k_0$, eqn. A8 can be simplified to

$$v(x) = \frac{E - 1}{(1+\kappa)E - 1} \cdot u = \frac{(E-1)(1+\kappa)}{(1+\kappa)E - 1} \cdot u_d \tag{A9}$$

At the column exit the linear velocity of the zone is $v = v(x = L)$ and this is the velocity at which the zone is eluted into the detector. Consequently, if additional gradient zone contraction were not taken into account, the following equation would be valid for time dispersion:

$$\sigma_t^2 = \frac{\sigma_L^2}{v^2(x = L)} = \frac{\int_0^L H_0(x)\,dx}{v^2(x = L)} \tag{A10}$$

where $H_0(x) = d\sigma_{L,0}^2/dx$ is the local plate height. In reality, because of the concentration gradient of the displacer along the column, the concentration point of the zone located at a distance $x - \sigma_L$ should migrate at a higher velocity than the centroid, and the concentration point $x + \sigma_L$ should migrate more slowly than the zone centre.

Let us now consider the change in the standard deviation of the peak σ_L after a small distance Δx is passed along the column only as a result of the difference between the velocities v (at the point with a coordinate $x + \sigma_L$) and v_x:

$$\Delta\sigma_L = (v_\sigma - v_x)\,\Delta t = \left(\frac{v_\sigma}{v_x} - 1\right)\Delta x \tag{A11}$$

At the point $x + \sigma_L$ the local composition of the

mobile phase will correspond to $\varphi(x+\sigma_L)=\varphi_0+B[t_x-(x+\sigma_L)/u_d]$. Using eqn. A7 for t_x, one may derive the following expression for the local capacity factor at the point $x+\sigma_L$:

$$k'_{(x+\sigma L)}=k_w 10^{-S\varphi(x+\sigma_L)}=\frac{E10^{SB\sigma_L(1+\kappa)/u}}{(\kappa/k_0)+E-1}$$

As usually $\kappa << k_0$, the last equation may be written in the simple form

$$k'_{(x+\sigma_L)}=\frac{E\cdot 10^{SB\sigma_L(1+\kappa)/u}}{E-1} \quad \text{(A12)}$$

Hence the velocity v_σ may be given as

$$v_\sigma=\frac{u}{1+k'_{(x+\sigma_L)}}$$

$$=u\cdot\frac{E-1}{E-1+E\kappa\cdot 10^{SB\sigma(1+\kappa)/u}} \quad \text{(A13)}$$

When σ_L is small, $10^{SB\sigma_L(1+\kappa)/u}$ may be approximated as $1-2.3SB\sigma_L(1+\kappa)/u$.

Applying eqn. A13 for v_σ and eqn. A9 for $v(x)$ and taking into account the above approximation, we obtain from eqn. A11 the following approximate equality:

$$\Delta\sigma_L=-2.3\cdot\frac{SB\kappa(1+\kappa)}{u}\cdot\frac{E}{E(1+\kappa)-1}\cdot\sigma_L\,\Delta x \quad \text{(A14)}$$

The corresponding change for dispersion is given by

$$d\sigma_L^2=2\sigma_L\,d\sigma_L$$

$$=-4.6\cdot\frac{SB\kappa(1+\kappa)}{u}\cdot\frac{E}{E(1+\kappa)-1}\cdot\sigma_L^2\,dx \quad \text{(A15)}$$

Correcting the local plate height for the front sharpening (eqn. A15), we obtain the following differential equation for real plate height in gradient chromatography:

$$H(x)=\frac{d\sigma_L^2}{dx}=H_0(x)-4.6\kappa\cdot\frac{SB}{u_d}\cdot\frac{E}{E(1+\kappa)-1}\cdot\sigma_{L,0}^2 \quad \text{(A16)}$$

where $H_0(x)$ and $\sigma_{L,0}^2$ are the plate height and dispersion at the point x, respectively, under the condition that gradient contraction is absent.

The solution of eqn. (A16) may be obtained explicitly if we use the dependence of HETP on k' given by eqn. 30. In this case the solution is

$$\sigma_t^2=0.434\cdot\frac{L}{u}\cdot\frac{1}{(E-1)^2}\left\{\frac{w(1+\kappa)^2+\alpha\kappa^2}{2r}(E^2-1)-\frac{2w(1+\kappa)(E-1)}{r}+2.3\omega\right\} \quad \text{(A17)}$$

where $E=10^{-SBL\kappa/u}$ and L is the column length. This solution was obtained taking into account that $\sigma_t^2=\sigma_L^2/v^2$ (eqn. A10) and $v(x=L)$ being given by eqn. A9.

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