ELSEVIER

Journal of Chromatography A, 753 (1996) 217–225

JOURNAL OF CHROMATOGRAPHY A

Peculiarities of gradient ion-exchange high-performance liquid chromatography of proteins[1]

N.I. Dubinina*, O.I. Kurenbin, T.B. Tennikova

Institute of Macromolecular Compounds, Russian Academy of Sciences, 199 004 St. Petersburg, Russia

Received 28 May 1996; revised 30 May 1996; accepted 4 June 1996

Abstract

Since the influence of column length on protein resolution in high-performance liquid chromatography (HPLC) is not clear, different viewpoints presented in the literature are analysed in detail. The influence of gradient steepness on the length of the working column part (X_0) or the part of a column in which the quasi-steady state is attained was studied. The equation for estimating the X_0 value was obtained for the general case of the retention model. It was shown that at steep gradients only a short part of the column is used as the working part on which all separation processes develop. The other part of a column is a ballast where the protein zone migrates in a regime of parallel transfer. These results form a theoretical basis for high-performance membrane chromatography. As was shown experimentally, this method makes it possible to perform protein separation at low gradient times with appropriate resolution, comparable with that of HPLC.

Keywords: Gradient elution; Column length; Proteins

1. Introduction

The behaviour of proteins in high-performance liquid chromatography (HPLC) columns is substantially different from that of small molecules. First, the process of separation is complex because of the three-dimensional protein molecule structure. Conformational changes in proteins in stationary or mobile phases sometimes take place during interactive types of HPLC, especially in the reversed-phase mode (RP-HPLC) [1,2]. The second peculiarity is irreversible binding of proteins with separation media. In this case rectangular adsorption isotherms occur when the salt concentration or concentration of an organic modifier is low. Nevertheless, it is considered that proteins in gradient chromatography are eluted from the column in agreement with the all or nothing principle for concentration changes [3–11]. However, in a real situation there is some elution window for a protein defined as the smallest range of displacement agent concentration in the mobile phase that would cause a protein to pass from the range of strong binding to that of weak binding. The multipoint character of protein binding causes this window to be quite narrow [12–16,18,19]. This situation is convenient for a good separation of proteins when a protein mixture has separate elution regions.

Third, the general plate theory is not applicable to

*Corresponding author.

[1] Presented at the 19th International Symposium on Column Liquid Chromatography, Innsbruck, 28 May–2 June 1995; partly presented at the 11th International Symposium on Preparative and Industrial Chromatography, Baden-Baden, 3–6 October 1994.

0021-9673/96/$15.00
PII S0021-9673(96)00545-6

protein separation. The effect of column length on the resolution factor R_s is more complicated than that for small molecules. The main conclusion is that very short columns and even flat macroporous membranes a few millimetres thick [16–19] can be used for the effective separation of proteins. However, so far there is little information about factors influencing column length. The most detailed analysis of the effect of column length on R_s and peak width has been given by Snyder [3,20], Yamamoto [21], and Koyama [15].

Equations for quantitative prediction of bandwidth and peak capacity in the RP-HPLC of proteins suggest that resolution R_s and peak height should be relatively insensitive to change in column length. Some investigators have observed comparable resolution for the columns 2–50 mm length, but other studies have shown that R_s generally decreases for very short columns [20]. Some interesting conclusions have been made using computer simulation of chromatographic separation. It has been shown that maximum peak capacity and resolution occur for longer columns when gradient time is allowed to increase. These observations suggest that there is an optimum column length giving maximum resolution for some preferable gradient time. Yamomoto et al. [21,22] have shown that resolution is proportional to the square root of column length when linear mobile phase velocity, the particle diameter and the slope of gradient, which is normalized to the column length are kept constant.

The approach of Belenktii et al. [23] to gradient chromatography of proteins is based on the concept of critical chromatography of synthetic polymers [24–28]. The main features of critical chromatography are as follows: at the critical point the exclusion entropy effect is compensated for by the enthalpy effect. Moreover, the Gibbs energy ΔG is $-RT \log K_d=0$, and the value of distribution constant K_d is unity. In isocratic elution all macromolecules after attaining the critical point are eluted at this value of K_d. In the case of gradient chromatography of proteins, the equation of zone migration was solved with respect to the distance passed by the zone and the distance at which K_d equal to unity was found [23]. Under this condition the velocity of the protein zone becomes equal to that of the displacer. Thus the definition of the critical distance was introduced, and the equation for it was obtained

$$X_0 = \lambda u/(SB) \quad (1)$$

where λ is the auxiliary parameter, u is the linear elution velocity, S is the dimensionless protein adsorption parameter (in reversed-phase mode), and B is the steepness of linear gradient.

This is the only study which makes it possible to estimate the critical length of a column in gradient HPLC of proteins. However, in chromatography for describing the moving boundary the approach not based on critical conditions is generally applied. This is the quasi-steady state model. The concept of the quasi-steady state of the zone boundary in frontal ion-exchange process was first considered by Shilov et al. [29], Rachinsky [30] and Glueckauf [31]. Then it was presented by Svenson [32] for isoelectric focusing, and by Sluterman and Elgersma [33] for chromatographic focusing. Yamamoto et al. [21] employed the model of the quasi-steady state for gradient protein elution.

According to this model, the zone spreading effects in the region of adsorption increase the protein peakwidth, but at the same time the zone compression effects caused by the flow tend to sharpen this peak during gradient elution. In the early elution period, the zone spreading effects predominate and the width of the protein zone increases. Since the contribution of zone compression increases with time, zone compression becomes greater with increasing zone width. Finally, zone compression effects may balance zone spreading effects. The width of protein zone does not increase any longer, i.e., the quasi-steady state is attained. It occurs when the K_d of the protein is equal to that of the displacer. We will denote K_d of protein in the quasi-steady state as K_d (st). In contrast to critical chromatography this value of K_d(st) is variable and may not be equal to unity.

The displacer concentration at which distribution constants of exchanging molecules are equal is very important. It reflects the intrinsic adsorption property of the protein. In the quasi-steady state the velocity of protein zone migration is equal to that of the displacer. When the protein zones move at equal velocities, i.e., the quasi-steady state is achieved for all protein zones, the distance between them does not change any longer and the constant R_s value is attained [21].

The case when the protein distribution constant is

equal to unity is evidently a particular case of a more general situation such as the quasi-steady state. Hence, it is actually necessary to develop the theory for the case of the quasi-steady state of a protein zone and to confirm it by experimental data.

As a part of the study on the specific features of protein gradient HPLC, the present paper describes the investigation directed to finding out (i) the interrelation between critical distance and the parameters of protein adsorption and gradient elution, and (ii) the influence of the ratio of the critical distance to column length on bandwidth and resolution.

Note that the terms of critical concentration and the critical distance mean only the attainment of the quasi-steady state. There is difference between the definition of the critical distance in critical chromatography and its definition in our case.

2. Theory

If the velocity of zone migration is a function of the mobile phase composition $\nu = uf(c)$ and the gradient function is known, then the differential equation of zone migration is given by

$$dx/dt = u\, f[c(t - x/u')] \tag{2}$$

where u is the elution velocity and u' is the displacer velocity.

The solution of this equation

$$t = \int_0^{t-x/u'} d\tau/\{1 - (1 + x)\, f[c(\tau)]\} \tag{3}$$

gives the dependence of the distance x passed by the zone during the time t.

Some special types of gradient and f(c) make it possible to carry out integration analytically and to find the solution of the Eq. (3).

For instance, the function $f(\varphi) = 1/(1 + k'_w e^{-S\varphi})$, which is sufficiently exact for RP-HPLC (here φ is the volume fraction of organic modifier), makes it possible to find this solution in the explicit form for linear gradient elution.

The critical distance X_0 (Eq. (1)) at which the zone velocity $\nu(x)$ becomes virtually identical to displacer velocity u' can be determined.

In this paper the theory for any case of functional dependencies f(c) and $c(t)$ is examined.

When x is equal to X_0, the velocity of zone migration is given by

$$\nu = \nu_0 = u'(1 - \epsilon) \tag{4}$$

where ϵ determines the degree of approximation ν to u'. In this case the velocity of zone migration at $x = X_0 + dx$ is determined as

$$\nu_0 + d\nu = u\, f(c(t_0 + dt - x/u')) \tag{5}$$

where t_0 is the time of reaching the point X_0. Consequently, we have

$$\nu_0 + d\nu = u\, f(c(t_0 - X_0/u' + dx(1/\nu_0 - 1/u')))$$

Expanding the function f(c) to Tailor's series about the point $\tau_0 = t_0 - X_0/u'$ and using only the first two expansion terms, we obtain

$$\nu_0 + d\nu = u(\tau_0) + u\, f'_c(C_c)c'_t(\tau_0)(1/\nu_0 - 1/u')dx \tag{7}$$

where f'_c and c'_t are derivatives, C_c is $C(\tau_0)$.

Taking into account the fact that $uf(\tau_0)$ is equal to ν_0 we obtain

$$d\nu = f'_c(C_c)c'_t(\tau_0)(u' - \nu)u/u'^2 dx \tag{8}$$

It follows from Eq. (8) that approximation ν to u' has exponential character and at high x value we have

$$\nu - u' = Ae^{-Dx} \tag{9}$$

where A is integration constant and D is determined by the equality

$$D = u/u'^2 f'_c(C_c)c'_t(\tau_0) \tag{10}$$

Bearing in mind that $u' = (1 + \kappa)$ it may be concluded that in the general case of arbitrary functional dependence of log k' on displacer composition the critical distance may be found from the equation

$$X_0 = \lambda u/(f'_c(C_c)c'_t(\tau_0)) \tag{11}$$

where λ depends on the ratio of the inner pore volume of the sorbent to the interparticle void volume of the column and the degree of approximation of ν to u', i.e., ϵ.

Applying this equation to the functional dependence f(c) for a stoichiometric model of protein retention

$$f(c) = 1/(1 + Kc^{-Z}) \tag{12}$$

in the case of linear gradient

$$c = c_0 + Bt \quad (13)$$

we have

$$X_0 = \lambda u C_c/(ZB) \quad (14)$$

This equation enables us to determine the critical distance, X_0, at which the quasi-steady state is attained.

3. Materials and methods

3.1. Proteins and reagents

Chymotrypsinogen A and cytochrome *c* (protein molecular mass standards) were obtained from Serva (Germany), pancreatic ribonuclease A (protein molecular mass standards) was purchased from Pharmacia (Sweden), egg lysozyme recrystallized three times (type B) was from Reakhim (Latvia). Eluent A was a 0.025 *M* phosphate solution adjusted to pH 6.5. Eluent B comprised 0.025 *M* phosphate and 0.5 *M* NaCl at pH 6.5. Protein solutions were prepared by dissolving the protein in buffer A at a concentration of 0.5 mg/ml.

A protein anion-exchange standard mixture (Bio-Rad Labs., CA, USA) was used for anion-exchange high-performance membrane chromatography (HPMC). The mixture consisted of myoglobin (from horse heart), conalbumin, chicken egg albumin and soybean trypsin inhibitor. Vial content (9 mg protein) was dissolved in 4 ml of an appropriate buffer (buffer A). Buffer A was a 0.01 *M* Tris solution pH 6.8, buffer B was a 0.6 *M* NaCl solution in buffer A adjusted to pH 6.8.

4. Separation media

Data were obtained with a strong cation-exchange column (Pharmacia Mono-S, HP 5/5, 50×5 mm I.D.). All chromatographic separations were carried out at 1 ml/min flow-rate and 20 °C.

Macroporous membranes were prepared by bulk polymerization in a mould described elsewhere [17] and cut to discs. Quick Disc cartridges in which chromatography proceeds, where a gift of Saulen-technik Knauer (Berlin, Germany). Standard chromatographic equipment consisted of a Rheodyne injection valve with a 200-μl loop, a cartridge containing a membrane 20 mm in diameter and 3 mm thick functionalized with diethylamino groups, as well as of two HPLC gradient pumps, and an UV detector (both from LKB, Bromma, Sweden).

5. Equipment

Standard equipment for the column cation-exchange HPLC (HPIEC) consisted of a Model 421 gradient programmer, two Model 110 pumps, an Altex Model 210 sample injection valve with a 20-μl loop, and a Model SPD-2AM photodiode array detector (Shimadzu, Kyoto, Japan), set to 225 nm. Chromatograms were recorded on a Kipp and Zonen BD recorder.

6. Chromatographic evaluation

The interparticle volume, V_0, was obtained by chromatography of ferritin (M_r=450 000) in 100% eluent B.

The retention factor k' and the linear velocity, u, were calculated with respect to the retention volume of ferritin.

The critical concentration value was obtained at the point of k' equal to $K_{d(st)}\ \kappa$. The value of κ was determined as the ratio of the intraparticle pore volume to the interparticle volume of the column.

The intraparticle volume, V_p, was determined as the difference between the void volume of a column and the interparticle volume.

The void volume of a column was determined by sodium azide chromatography.

Constant, Z, characterizing the electrostatic interaction was determined as a slope of a plot of log k' versus log c.

7. Results and discussion

Belenkii et al. [23] have examined only RP-HPLC and have used the following equation for the retention factor k'

$$\log k' = \log k_w - S\varphi \quad (15)$$

where φ is the volume fraction of organic solvent, and S is the dimensionless protein adsorption parameter.

At the same time in ion-exchange (IEC), and hydrophobic interaction (HIC) as well as in RP-HPLC at $\varphi > 0.30$, the dependence of retention factor k' on the concentration of the displacer may be described according to the stoichiometric displacement model of Geng and Regnier [34] by the general equation

$$\log k' = \log K - Z \log c \quad (16)$$

where c is the molar concentration of the displacer, and Z is the constant characterizing protein interaction with the chromatographic separation surface.

The experimental dependence of k' on the composition of the mobile phase is given in Fig. 1. The k' dependencies for different proteins do not intersect at one point which corresponds to the critical concentration as was observed in critical chromatography of synthetic polymers and as was supposed and obtained for some proteins [23]. Results similar to ours have been presented by Hearn et al. [35] in numerous experiments.

If the k' dependencies intersected at this point, resolution of proteins would not be good at the quasi-steady state. Elution windows of proteins would overlap heavily because the critical concentration for different proteins is the same. This means that in the quasi-steady state all proteins emerge from the column at this displacer concentration. The situation is very unfavourable for separation.

The equation for protein resolution presented by Yamamoto et al. [21,22] is consistent with our results and suggestions. According to this equation, R_s, is proportional to the difference in concentrations of the displacer at which zones appear at the exit of a column. If the quasi-steady state is attained and critical concentration is the same for all proteins, R_s will be equal to zero.

The lengths of a column, calculated according to Eq. (14), at which the quasi-steady state is attained for a given gradient time are presented in Fig. 2. The values of Z were obtained for all proteins from experimental dependencies of log k' on log C by the least squares method. As can be seen for steep gradients or low gradient time (1–5 min), only a short part of the column is used for developing the chromatographic process. We call it the working column part, the partitioning length, or the critical distance. The remaining part of the column is a ballast in which the protein zones move in the

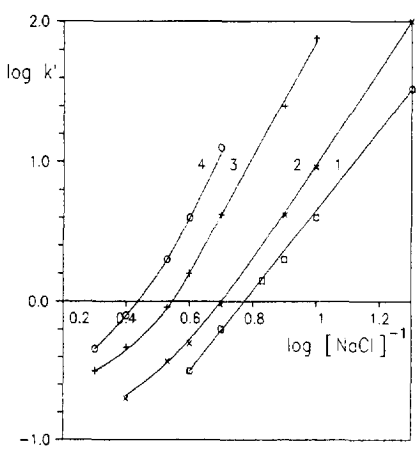

Fig. 1. Dependence of log k' on log [NaCl]$^{-1}$. The plots were derived from isocratic elution data at a flow-rate of 1 ml/min for (1) ribonuclease A, (2) chemotrypsinogen A, (3) cytochrome c, and (4) lyosozyme. Column chromatography.

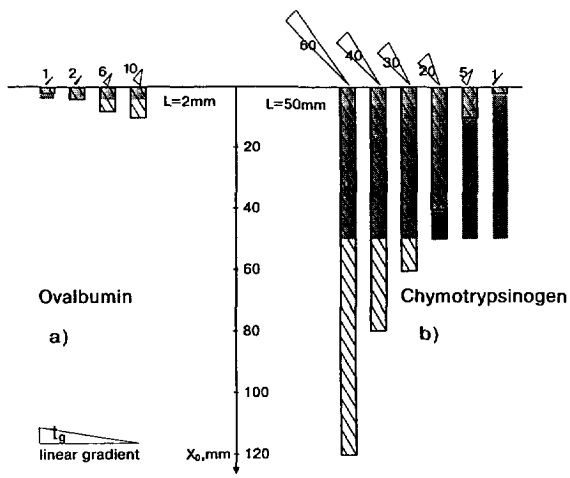

Fig. 2. Effect of gradient time on the working part of (a) the membrane and (b) the column. Dashed part represents working part of the membrane or the column, shadow part corresponds to the column length or to the membrane thickness. Triangles with numbers are attributed to gradient time, t_g, one side of triangle is proportional to t_g. X_0 is column (membrane) critical length.

regime of parallel transfer without considerable spreading.

If spreading takes place, one can take into consideration only that of the gradient itself. However, its influence cannot be significant under these conditions. At shallow gradients or high gradient times (40–90 min) the working part of a column for developing the chromatographic process and the achievement of the quasi-steady state is substantially greater than the length of the column used. The proteins emerge from the column at a high protein distribution constant K_d and, accordingly at a high retention factor k'.

Fig. 3 shows the dependence of σ_t on X_0/L. When X_0/L is approximately equal to unity (geometrical length of the column is approximately equal to the working column part) a plateau region appears. Subsequently σ_t increases rapidly with increasing X_0/L ratio since zones emerge at the column exit at a high k' value. When X_0/L decreases from unity, σ_t also decreases; this is due to the compression effect of the zone at steeper gradients. Our results are different from those predicted earlier [23] where the gradient compression effect is not taken into account. It has been considered in [23] that protein zone undergoes progressive spreading after passing the critical distance.

Hence, the vicinity of X_0/L, approximately equal to unity, divides the plot into three regions. The first one is a region where the quasi-steady state takes place (X_0 is less than unity), the second region is the transition region and, finally, there is a part of the curve which corresponds to a strong protein molecular contact with the sorbent surface. In the third part very large dispersion of the protein zones is observed, and this zone is eluted at a high k' value since X_0/L is greater than unity. The same tendency characterizes HPMC.

Since the value of X_0 depends on the gradient steepness B ($B = 1/t_g$), the dependence of peak width σ_t on X_0/L reproduces exactly the character of the dependence of σ_t on t_g.

The dependence of the distance between two adjacent peaks Δt on t_g or on X_0/L is similar to that of σ_t on X_0/L and increases in the region where X_0/L is greater than unity (Fig. 4). According to Yamomoto et al. [21] and Belenkii et al. [23], the dependence of the retention time on C_R may be expressed as follows:

$$t_R = \text{const} + (c_R - c_0)/B \tag{17}$$

where c_R is the displacer concentration at t_R and c_0 is that when the column is initially equilibrated.

Consequently, the difference in the retention time of two zones of proteins in gradient elution Δt can be written as

$$\Delta t = (c_{R_2} - c_{R_1})/B = (c_{R_2} - c_{R_1})t_g \tag{18}$$

where c_{R_1} and c_{R_2} are the displacer concentrations at t_{R_1} and t_{R_2} for two proteins, respectively.

In the quasi-steady state

$$\Delta t = (C_{c_1} - C_{c_2})/B = \text{const}\, t_g \tag{19}$$

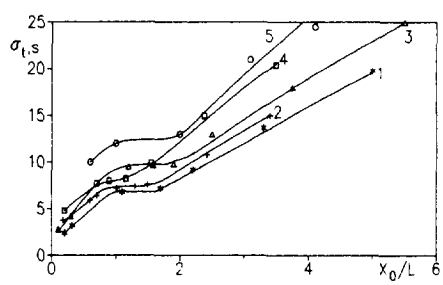

Fig. 3. Effect of X_0/L ratio on peak bandwidth σ_t for HPLC (1–4) and HPMC (5). Proteins: (1) cytochrome *c*, (2) ribonuclease A, (3) lysozyme, (4) chymotrypsinogen A, (5) ovalbumin. Conditions: linear gradient from 0 to 100% eluent B, flow-rate 1 ml/min for column separation and 5 ml/min for membrane separation.

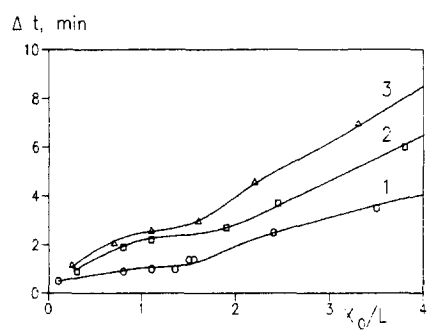

Fig. 4. Effect of X_0/L ratio on the difference in retention time Δt of the pairs (1) ribonuclease A–chymotrypsinogen A, (2) cytochrome *c*–lysozyme, and (3) chymotrypsinogen A–cytochrome *c*. Conditions as in Fig. 3.

As can be seen from Eq. (19), in this state Δt is proportional only to t_g. Just as in the previous case, the appearance of a plateau at X_0/L which is approximately equal to unity is due to the transition to the quasi-steady state.

The dependence of R_s on X_0/L has a typical character similar to that of R_s on t_g (Fig. 5a,b).

Two questions arise from the foregoing. Is it advantageous to work in the quasi-steady state? Because the quasi-steady state is realized at X_0 less than L, it occurs at steep gradients or on long columns. As the aim of any separation is to obtain good resolution, it is not important in which state the separation is performed. However, in optimizing the conditions of gradient elution the length of a column must be taken into account. Hence, the column length is the second question. In other words, which column length is required for improved resolution? It is understandable why in some cases an increase of column length is not of any use [15,20–22]. This can occur when the quasi-steady state is already attained. Then with increasing column length the column part where the zone moves in a regime of parallel transfer increases and improved resolution is not observed. To improve resolution, it is necessary to increase the gradient time.

When the quasi-steady state is not achieved, R_s increases with column length.

Short columns may be effectively used when elution windows of separated proteins do not overlap, i.e., critical concentration is different for the different proteins. It was confirmed in our study by several separations both on columns and on membranes. At very steep gradients (5 min and less) and, correspondingly, at small partitioning length X_0 the separation of the protein mixture was good (Fig. 6). All proteins have similar physico-chemical properties; nevertheless they have virtually non-overlapping elution windows (Fig. 1).

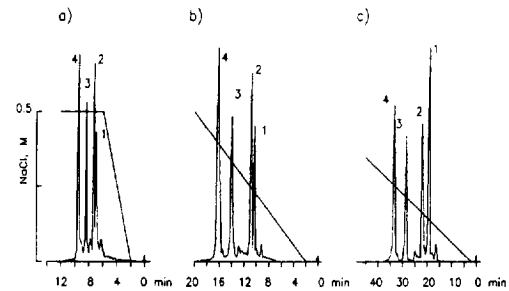

Fig. 6. Effect of gradient time on protein separation in HPIEC: (1) ribonuclease A, (2) chymotrypsinogen A, (3) cytochrome *c*, (4) lysozyme, gradient from A to 100% B: (a) 5 min, (b) 20 min, (c) 60 min.

These results make it possible to use high-performance membrane chromatography for the separation of proteins with appropriate resolution and low gradient times (Fig. 7). It is clear that the linear mobile phase velocity u is much lower in the case of HPMC because of much greater cross section that is

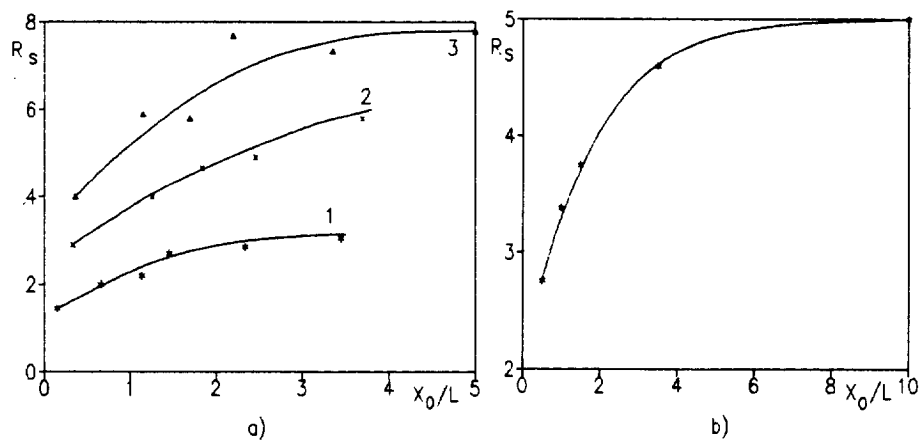

Fig. 5. Resolution as a function of X_0/L ratio for protein pairs (a) in column chromatography: (1) ribonuclease A–chymotrypsinogen A, (2) cytochrome *c*–lysozyme, (3) chymotrypsinogen A–cytochrome *c*; (b) in membrane chromatography: ovalbumin–soybean trypsin inhibitor. Conditions as in Fig. 3.

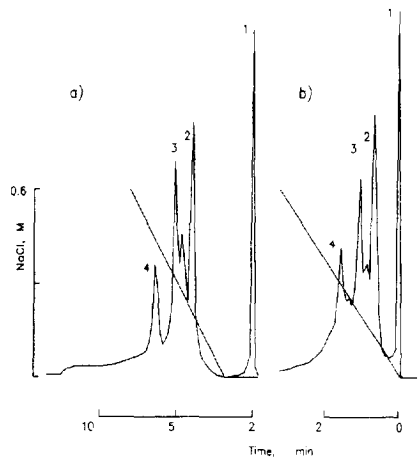

Fig. 7. HPMC separation of (1) myoglobin, (2) conalbumin, (3) ovalbumin and (4) soybean trypsin inhibitor in the same volume of linear gradient (30 ml) under various flow-rates: (a) 5 ml/min, (b) 10 ml/min.

lower the value of X_0. However, when separating layer is 1–3 mm, it is possible to use ultra high working flow-rates, up to 15–30 ml/min, at extremely low back pressure and very short gradient times.

The main idea discussed above provided a possibility of controlling protein resolution from the point of view of separation layer length (working part of column). Since the same approaches were used for both HPLC and HPMC, it became possible to compare these methods (Fig. 8). It was found that the same resolution, R_s, for two adjacent proteins in membrane chromatography corresponds to much smaller length of the separation layer X_0 and very short analysis time. It should be noted that this figure serves only for comparison of HPLC and HPMC.

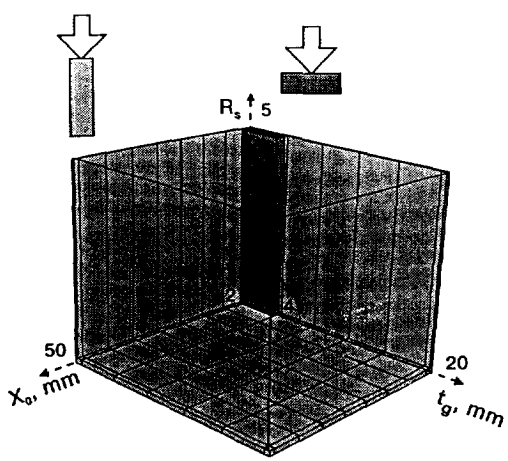

Fig. 8. Comparable separation of protein pairs: chymotrypsinogen A–cytochrome *c* (HPLC-light shadow part), ovalbumin–soybean trypsin inhibitor (HPMC-dark shadow part). Conditions as in Fig. 3.

8. Conclusions

It is shown that at steep gradients only a short part of a column is used as the working part or the part in which the quasi-steady state of the protein zone is attained. The other part of the column may be considered as a ballast where the protein zone migrates in the regime of parallel transfer without substantial spreading. Taking into account good resolution of protein zones even at very steep gradients and the small thickness of the adsorbing layer, HPMC may be considered as more appropriate. It was shown that membrane chromatography can be used to separate proteins with good resolution in a short time.

9. Symbols

B	steepness of linear gradient (Eq. (13))
c	mobile phase salt concentration (M)
C_c	critical concentration of the displacer salt (M)
c_0	initial value of c in ion-exchange gradient elution (M)
c_R	displacer salt concentration at t_R (M)
k'	retention factor (dimensionless)
k_w	retention factor in water as a mobile phase (dimensionless)
K	ion-exchange equilibrium distribution constant (Eq. (16))
K_d	distribution constant
$K_{d(st)}$	distribution constant at the quasi-steady state
L	column length (cm)
R_s	resolution function
S	dimensionless protein adsorption parameter (in RP-HPLC) (Eq. (15))

t	time after start of gradient and sample injection
t_g	gradient time
t_R	retention time
u	mobile phase velocity (cm/s)
u'	displacer velocity (cm/s)
V_p	inner pore volume of the sorbent
ν	migration velocity of the zone maximum
χ	distance along the column
X_0	critical distance defined by Eq. (1) or Eq. (14)
Z	the effective charge on the solute ion divided by the charge on the mobile phase ion (in ion-exchange)
φ	the volume fraction of organic solvent in the mobile phase (in RP-HPLC)
κ	the ratio of the inner pore volume of the sorbent to the interparticle void volume of the column
λ	auxiliary parameter (Eq. (11))
σ_t	peak standard deviation in time units

Acknowledgments

The authors gratefully acknowledge the financial support from the International Science Foundation (grant No. R5S000).

References

[1] S.A. Cohen, K. Benedek, Y. Tapuhi, J.C. Ford and B.L. Karger, Anal. Biochem., 144 (1985) 275.
[2] J.P. Andrade, in J.P. Andrade (Editor), Principles of Protein Adsorption/Surface and Interfacial Aspects of Biomedical Polymers/, Vol. 2, Plenum Press, New York, 1985, pp. 1–80.
[3] L.R. Snyder and M.A. Stadalius, in Cs. Horváth (Editor), High-performance Liquid Chromatography–Advances and Perspectives, Vol. 4, Academic Press, New York, 1987, p. 195.
[4] J.D. Pearson, W.C. Mahoney, M.A. Hermodson and F.E. Regnier, J. Chromatogr., 207 (1981) 15.
[5] F.E. Regnier, Science, 222 (1983) 245.
[6] X. Geng and F.E. Regnier, J. Chromatogr., 296 (1984) 15
[7] D.W. Armstron and R.E. Boehm, J. Chromatogr. Sci., 22 (1984) 378.
[8] J.L. Fausnaugh, L.A. Kennedy and F.E. Regnier, J. Chromatogr., 387 (1987) 21.
[9] K.H. Bui, D.W. Armstrong and R.E. Boehm, J. Chromatogr., 288 (1984) 15.
[10] R.E. Boehm, D.E. Martire, D.W. Armstrong and K.H. Bui, Macromolecules, 17 (1984) 4003.
[11] N.J. Dubinina, A.A. Seleznyova, G.E. Elkin and G.V. Samsonov, Vysokomol. Soedin., 15B (1983) 517.
[12] A. Velayudhan and Cs. Horváth, J. Chromatogr., 443 (1988) 13.
[13] F.E. Regnier and J. Mazsaroff, Biotechnol. Prog., 3 (1987) 22.
[14] W. Kopaciewicz, M. Rounds, J. Fausnaugh and F.E. Regnier, J. Chromatogr., 266 (1983) 3.
[15] J. Koyama, J. Nomura, Y. Shiojima, Y. Ohtsu and J. Horri, J. Chromatogr., 625 (1992) 217.
[16] J.L. Coffman, D.K. Roper and E.N. Lightfoot, Bioseparation, 4 (1994) 183.
[17] T.B. Tennikova, B.G. Belenkii and F. Svec, J. Liq. Chromatogr., 13 (1990) 63.
[18] T.B. Tenniova, M. Bleha, F. Svec, T.V. Almazova and B.G. Belenkii, J. Chromatogr., 555 (1991) 97.
[19] T.B. Tennikova and F. Svec, J. Chromatogr., 646 (1993) 279.
[20] W.G. Bulton, K.D. Nugent, T.K. Slattery, B.A. Summers and L.R. Snyder, J. Chromatogr., 443 (1988) 363.
[21] S. Yamamoto, K. Naganishi and R. Matsuno, Ion-Exchange Chromatography of Proteins, Marcel Dekker, New York, Basel, 1988, p. 94.
[22] S. Yamomoto, M. Nomura and Y. Sano, J. Chromatogr., 409 (1987) 101.
[23] B.G. Belenkii, A.M. Podkladenko, O.I. Kurenbin, V.G. Maltsev, D.G. Nasledov and S.A. Trushin, J. Chromatogr., 645 (1993) 1.
[24] B.G. Belenkii, E.S. Gankina, M.B. Tennikov and L.Z. Vilenchik, Dokl. Akad. Nauk SSSR, 231 (1976) 1147.
[25] M.B. Tennikov, P.P. Nefedov, M.A. Lazareva and S.Ya. Frenkel, Vysokomol. Soedin., 19A (1977) 657.
[26] A.A. Gorbunov and A.M. Skvortsov, Vysokmol. Soedin., 30A (1988) 895.
[27] B.G. Belenkii, Pure Appl. Chem., 51 (1979) 1519.
[28] A.M. Skvortsov, E.S. Gankina, M.B. Tennikov and B.G. Belenkii, Vysokomol. Soedin., 20A (1978) 678.
[29] N.A. Shilov, L.K. Lepn and C.A. Voznesenskii, Zhur. Rus. Fiz. Khim. Obshch., 61 (1929) 1107.
[30] V.V. Rachinski, Zh. Fiz. Khim., 31 (1957) 444.
[31] E. Glueckauf and J. Coates, J. Chem. Soc., (1947) 1315.
[32] H. Svensson, Acta Chem. Scand., 15 (1961) 325.
[33] L.A.Æ. Sluyterman and O. Elgersma, J. Chromatogr., 150 (1978) 17.
[34] X. Geng and F.E. Regnier, J. Chromatogr., 332 (1985) 147.
[35] M.T.W. Hearn, A.N. Hodder and M.I. Aguilar, J. Chromatogr., 443 (1988) 97.