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High-performance membrane chromatography: highly efficient separation method for proteins in ion-exchange, hydrophobic interaction and reversed-phase modes

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ABSTRACT

High-performance membrane chromatography (HPMC) is a very effective chromatographic method in which all the mobile phase flows through the separation medium. The effects of process variables such as concentration of displacement agent, flow-rate and gradient slope on HPMC separations in the ion-exchange, hydrophobic interaction and reversed-phase modes were studied using model protein mixtures. The basic relationships characterizing column HPLC also apply in HPMC. Whereas the efficiency of the HPMC membrane does not depend on flow-rate, the resolution increases with increasing gradient volume. Separations obtained with a continuous linear gradient were used for the design of a stepwise gradient profile which decreases the consumption of both time and mobile phase in separations of proteins. According to calculations, the protein diffusivity enhanced by the convective flow through the membrane is about four orders of magnitude higher than the "free" diffusivity of the protein in the stagnant mobile phase located in the pores of a standard separation medium. This considerably speeds up the process and improves the efficiency of the separation.

INTRODUCTION

High-performance liquid chromatography (HPLC) revolutionized analytical chemistry by facilitating very rapid and efficient separations and the detection and determination of the components of virtually any mixture. The separation, isolation and purification of biopolymers is very important for their effective application. The analytical and preparative HPLC separations of individual biological macromolecules from their mixtures with both low- and high-molecular mass compounds has been reviewed several times [1–6].

At present, most chromatographic separations are carried out in columns packed almost exclusively with bead-shaped particles. As the technology of bead preparation has been known for more than two decades [7,8], current research is focused on the design of new shapes [9].

Traditional membranes were introduced into affinity chromatography in 1988 [10–18]. Ion-exchange cellulose membranes stacked in a cartridge also gave good results in the separation and purification of proteins [19–23]. The recently introduced high-performance membrane chromatography (HPMC) combines the advantages of both membrane technology (simple scale-up, low pressure drop across a membrane) and

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column chromatography (high selectivity and efficiency of separation, high loading capacity) [24–28]. The efficiency of HPMC has been demonstrated in a number of protein separations in ion-exchange, hydrophobic interaction and affinity chromatographic modes. Quick Disc cartridges using HPMC technology are commercially available [29].

HPMC is characterized by high efficiency and rapid separation. The effects of some variables, such as membrane thickness, cartridge construction and surface chemistry, on HPMC have been reported in previous papers [24–28]. This paper gives a quantitative description of the separation process in a macroporous membrane and optimization of separation conditions for different modes of HPMC.

EXPERIMENTAL

Preparation of membranes

Macroporous membranes were prepared by free radical copolymerization of a mixture containing, a monovinyl monomer [glycidyl methacrylate (GMA), octyl methacrylate, dodecyl methacrylate or styrene (ST)], a divinyl monomer [ethylene dimethacrylate (EDMA) or divinylbenzene [DVB)], an initiator [azobisisobutyronitrile; 1% (w/w) with respect to the monomers] and porogenic diluents (cyclohexanol and dodecanol) in a heated mould [25]. The polymer sheets were consecutively washed with methanol, benzene, methanol and water.

From the resulting 2–3-mm thick flat sheet of poly(glycidyl methacrylate–co-ethylene dimethacrylate) (50:50, v/v) discs about 20 mm in diameter were cut, modified with diethylamine [30] and placed in specially designed cartridges, which were a kind gift from Säulentechnik Knauer (Berlin, Germany) [29]. Discs cut from poly(glycidyl methacrylate–co-dodecyl methacrylate–co-ethylene dimethacrylate) and poly(glycidyl methacrylate–co-octyl methacrylate–co-ethylene dimethacrylate) (both 45:15:40, v/v/v) sheets were treated with 0.1 mol/l sulphuric acid at 80°C for 5 h. The epoxide groups of the glycidyl methacrylate units yield two adjacent hydroxyls and the hydrophilicity of the original matrix increases. Poly[styrene–co-divinylbenzene (tech.)] (50:50, v/v) membranes were cut and used without any additional treatment.

Chromatography

Mobile phase gradients were produced by an HPLC gradient pump (LKB) with a maximum flow-rate of 10 ml/min. The programmed and detected gradients were found to be almost identical. Samples were injected through a Rheodyne C7125 valve loop injector (200 μl). The separation was monitored using a dual variable-wave length UV detector (LKB). A detailed description of the experiments has been published elsewhere [25].

Protein anion-exchange standard mixture (Bio-Rad Labs.) containing horse heart myoglobin, conalbumin, Chicken egg albumin and soybean typsin inhibitor was used as a model mixture in ion-exchange chromatography. Chymotrypsinogen A (type II, from bovine pancreas), myoglobin and chicken egg albumin were purchased from Sigma and lysozyme (from hen egg white) and ribonuclease A (from bovine pancreas) from Boehringer Mannheim. All these proteins were used for the hydrophobic interaction chromatographic tests. Reversed-phase membranes were tested with a mixture consisting of chicken egg albumin and human serum albumin.

RESULTS AND DISCUSSION

Current knowledge of gradient elution HPLC clearly supports the fact that the column length has only a small effect on the resolution of large molecules such as proteins in any mode of retentive chromatography [31,32]. The characteristics of separation efficiency such as peak capacity (PC), resolution factor (R_s) and chromatographic band spreading expressed as band width in time or volume units (σ) or as peak height (PH) are functions of gradient time (t_G), particle size of packing (d_p), flow-rate (F) and solute diffusion coefficient (D_m). According to the theory [33]:

$$PC \approx R_s \approx t_G^{1/2} F^0 L^0 d_p^{-1} \quad (1)$$

$$1/\sigma_t \approx PH \approx t_G^{-1/2} F^{-1} L^0 d_p^{-1} \quad (2)$$

where L is the column length.

Although the column length does not affect the separation, it can alter the actual gradient shape and complicate the optimization of a chromatographic separation. Two variables are essential in elution chromatography: the capacity factor (k' or $\bar{k}$) and the chromatographic band spreading (σ). These are included in all basic expressions for both isocratic and gradient HPLC. More attention should be paid to σ as it not only depends on the kinetics of interactions between the solute and stationary phase but also includes contributions of both diffusion and instrumentation quality.

Comparison of column and membrane chromatography

Although the similarity of HPMC and HPLC has already been documented [24,25], there are also some substantial differences between the two techniques. For example, the chromatographic columns are typically several centimetres long. Therefore, the compositions of the mobile phase at the inlet and outlet of the column during a gradient elution are different and a gradient is formed also inside the column; the k' values for a particular compound differ along the column. An average capacity factor $\bar{k}$, defined as the value of k' when the band has moved half way through the column, has to be used for gradient elution [34]. The "length" of a membrane, which is actually the thickness of the membrane across which the mobile phase flows, is only a few millimetres. Therefore, a mobile phase gradient within the membrane is hardly conceivable at standard flow-rates. The column dead volume depends on the quality of the packing technique and exceeds 40% of the total column volume. In contrast to the columns packed with individual particles, the macroporous membrane is a single body and has no void volume of that kind. If part of the membrane pore volume contained in very large pores were to be taken as a "dead volume", it still would be very small in comparison with the packed column void volume. Moreover, the membrane dead volume probably equals the extra-membrane volumes. The total pore volume of a membrane 2 mm thick, 25 mm in diameter and of 50% porosity is 0.6 ml. A similar pore volume is present in a 120×4 mm I.D. column well packed with beads of 50% porosity. However, the interparticular void volume of such a column represents an additional 0.6 ml. This doubles the column dead time and increases the retention time of a non-retained compound. Owing to the differences, some equations describing gradient elution separation in a column may not completely apply in HPMC. However, we decided to operate with k' and σ determined experimentally from chromatographic data or calculated according to equations derived for HPLC anyway, as the differences seems not to be in the art but more likely in the degree.

Effect of the mobile phase gradient in HPMC

Important factors influencing the separation in gradient elution chromatography generally are the gradient volume V_G and gradient steepness B, defined by

$$B = (c_f - c_0)/V_G = \Delta c/V_G \qquad (3)$$

where $\Delta c = c_f - c_0$ is the difference between the final and initial concentrations of the displacement agent in the eluent. Fig. 1 shows the effects of gradient variables on the HPMC separation of a model protein mixtures in ion-exchange (IEC, Fig. 1a), hydrophobic interaction (HIC, Fig. 1b) and reversed-phase (RPC, 1c) modes.

The retention volume V_R depends in standard column chromatography on the gradient slope and, after substitution for B from eqn. 3, on the gradient volume:

$$\log V_R = a - b\log B = a' + b\log V_G \qquad (4)$$

where a, a' and b are constants characteristic of the gradient shape. The straight lines shown in Fig. 2 confirm that membrane chromatography obeys the same rules as HPLC. The gradient slope provides an effect on the band width σ particularly in the range of small B (Fig. 3), as documented by the HPMC separation of the protein pairs conalbumin–soybean typsin inhibitor in the IEC mode (Fig. 3a) and lysozyme–chymotrypsinogen in the HIC mode (Fig. 3b). The resolution factor of the proteins, R_s, decreases with increase in gradient steepness in

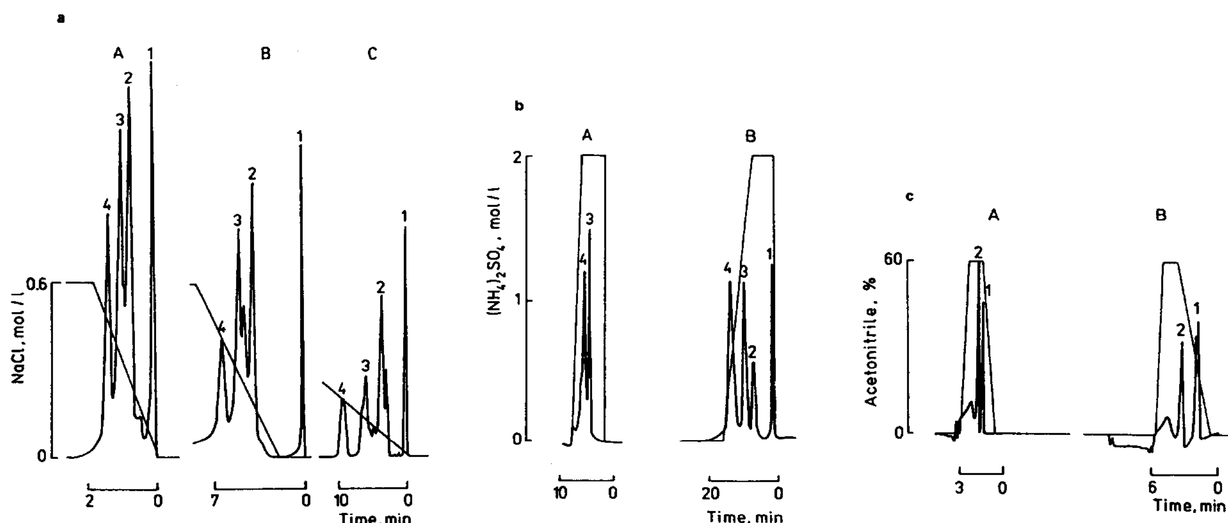

Fig. 1. Effect of various gradient volumes, V_G, on the separation of protein mixtures by (a) anion-exchange, (b) hydrophobic interaction and (c) reversed-phase high-performance membrane chromatography. (a) GMA–EDMA (50:50) modified membrane containing 1 mmol/g DEA groups, mobile phase 0.01 mol/l Tris–HCl buffer solution (pH 7.6), gradient from 0 to 0.6 mol/l NaCl in the buffer, gradient time (A) 2, (B) 6 and (C) 15 min, flow-rate 5 ml/min. Peaks: 1 = myoglobin; 2 = conalbumin; 3 = chicken egg albumin; 4 = soybean trypsin inhibitor. (b) Dodecyl methacrylate–GMA–EDMA (15:35:50) membrane, mobile phase 5 vol.% of 2-propanol in 0.02 mol/l phosphate buffer (pH 6.8), gradient from 2 to 0 mol/l $(NH_4)_2SO_4$ in the buffer, gradient time (A) 4 and (B) 10 min, flow-rate 3 ml/min. Peaks: 1 = myoglobin; 2 = ribonuclease A; 3 = lysozyme; 4 = chymotrypsinogen. (c) ST–DVB (50:50) membrane, mobile phase gradient from 0 to 60 vol.% aqueous acetonitrile, flow-rate 6 ml/min, gradient time (A) 2 and (B) 5 min. Peaks: 1 = chicken egg albumin; 2 = human serum albumin.

both IEC and HIC (Fig. 4). However, the band width of chymotrypsinogen eluted with a strong eluent in the HIC mode does not depend on the gradient slope.

An increase in the gradient volume in all the chromatographic modes tested improves the resolution up to the point beyond which even the single compound band splits into several peaks (Fig. 5). This may reflect the inhomogeneity of the solid surface of the separation medium, resulting in different interaction energies between the attached proteins and the surface or different accessibility of interacting areas.

In gradient elution chromatography, the capacity factor k' generally depends on the composition of the mobile phase. For ion-exchange chromatography,

$$\log k' = \log K - Z \log C \quad (5)$$

where K is a constant that includes the equilibrium formation constant, the phase ratio and the bound solute concentration, Z is the number of interaction sites for a particular solute and stationary phase and C is the actual total concentration of salts in the mobile phase [31]. The Z and log K data, calculated according to eqn. 5 from a series of isocratic membrane chromatographic runs, is summarized in Table I. Both Z and log K are smaller in HPMC than those obtained in HPLC under similar conditions [32].

Effect of flow-rate, gradient time and gradient profile

Flow-rate and gradient time are independent variables in gradient elution chromatography. Fig. 6 shows the effect of flow-rate on σ_v at constant gradient time and gradient volume in IEC with changing gradient volume and time, respectively. The band width decreases dramatically as the flow-rate increases, particularly at very low flow-rates. Similar effects were also observed in HIC HPMC. This is in contrast to gradient elution HPLC, in which a decrease in flow-rate may cause either a decrease or an increase in the resolution as a result of the

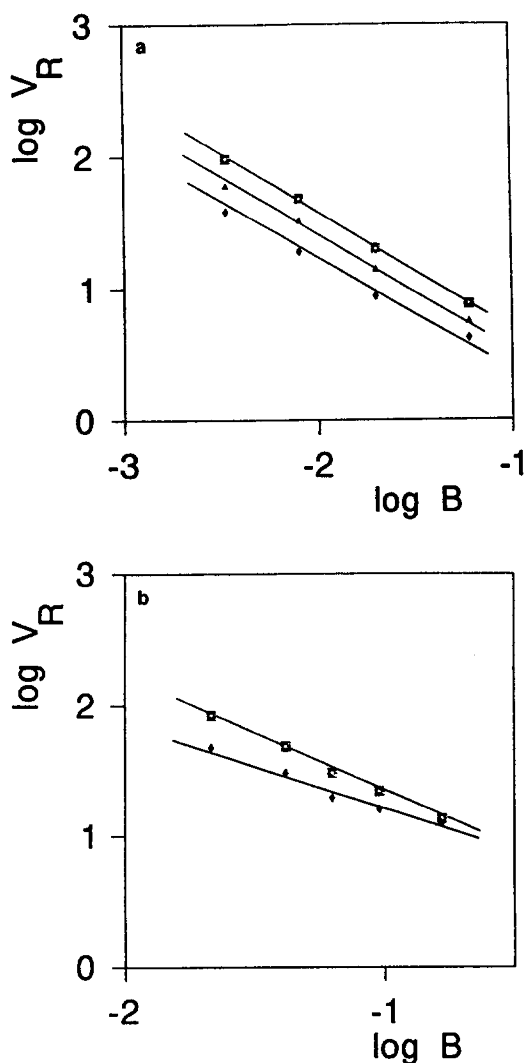

Fig. 2. Effect of the gradient slope B on the retention volume V_R for (a) anion-exchange HPMC of (◆) conalbumin, (▲) chicken egg albumin and (■) soybean trypsin inhibitor and (b) hydrophobic interaction HPMC of (◆) lysozyme and (■) chymotrypsinogen A.

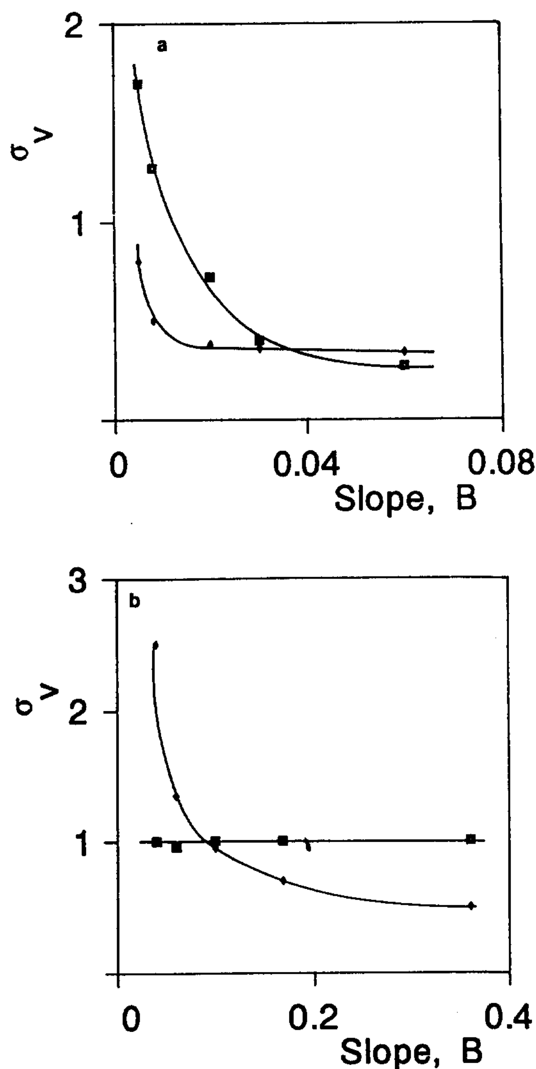

Fig. 3. Effect of the mobile phase gradient slope B on band spreading σ_V in (a) anion-exchange HPMC of (◆) conalbumin and (■) soybean trypsin inhibitor and (b) hydrophobic interaction HPMC of (◆) lysozyme and (■) chymotrypsinogen A.

trade-off between the increase in N and the decrease in k' [31].

Experiments revealed that the height equivalent to a theoretical plate as determined with myoglobin and acetone is about 0.4 mm (not taking in account the extra-column volume effects) and the efficiency does not change with flow-rate (Fig. 7). As the membrane efficiency does not depend on the linear flow velocity in range 0.04–1 cm/min, a decrease in separation quality occurs at higher flow-rates on account of the increase in k'. The calculated difference in composition of the mobile phase at the inlet and outlet of the cartridge (the mobile phase gradient within a 2 mm thick membrane) at constant gradient volume is 20% at 0.3 ml/min but only 2% at 3 ml/min. In the former instance k' may vary across the membrane whereas in the latter it remains almost the same in any part of the membrane. If the gradient time is constant, the

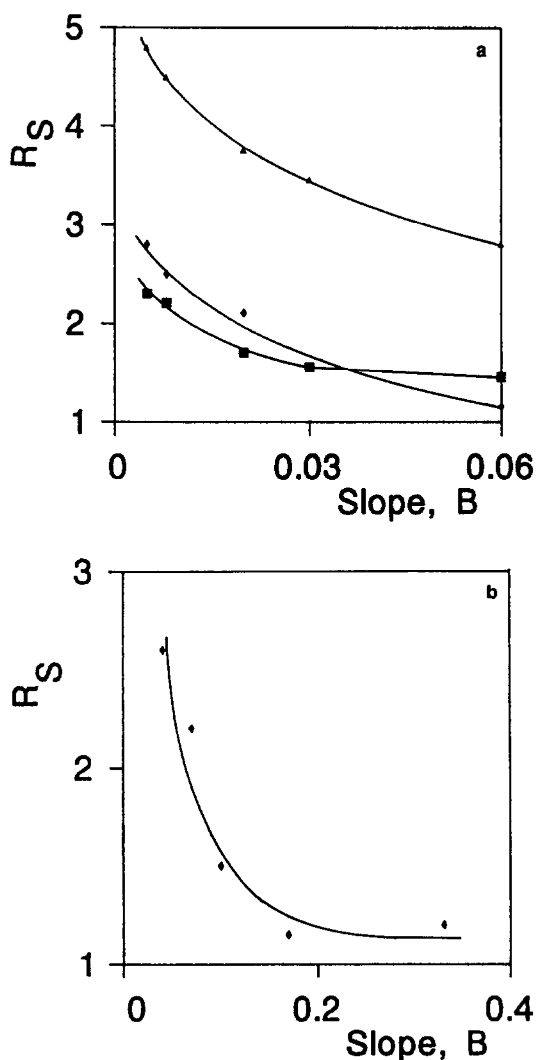

Fig. 4. Effect of the mobile phase gradient slope B on peak resolution R_s for (a) anion-exchange HPMC separation of the pairs (◆) conalbumin–chicken egg albumin, (■) chicken egg albumin–soybean trypsin inhibitor and (▲) conalbumin–soybean trypsin inhibitor and (b) hydrophobic interaction HPMC separation of (◆) lysozyme and chymotrypsinogen A.

gradient across the membrane does not change with the flow-rate and σ_v also remains constant. The effect of gradient steepness is similar to that observed in ion-exchange and reversed-phase HPLC [31,34,35].

The step-by-step optimization of the HPMC separation conditions results in a gradient the slope and lower and upper limits of which provide for good resolution at a chosen flow-

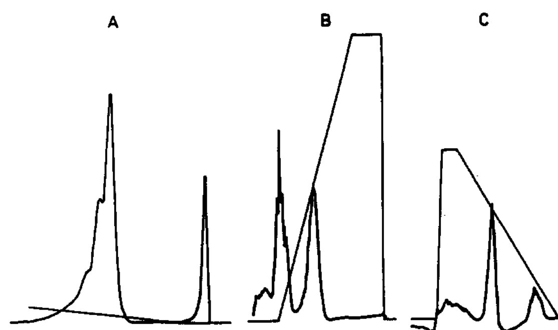

Fig. 5. Effect of increasing gradient volume on peak splitting. (a) Anion-exchange HPMC, gradient volume 150 ml; (b) hydrophobic interaction HPMC, gradient volume 90 ml; (c) reversed-phase HPMC, gradient volume 90 ml. Conditions as in Fig. 1.

TABLE I

CONSTANTS OF EQUATION 5 FOR PROTEINS IN ION-EXCHANGE HIGH-PERFORMANCE MEMBRANE CHROMATOGRAPHY

Protein	Log K^a	Z^a
Conalbumin	1.69 (4.31)	1.78 (2.39)
Chicken egg albumin	2.51 (6.39)	2.38 (6.66)
Soybean trypsin inhibitor	2.15	1.85

[a] Data in parentheses are from ref. 35.

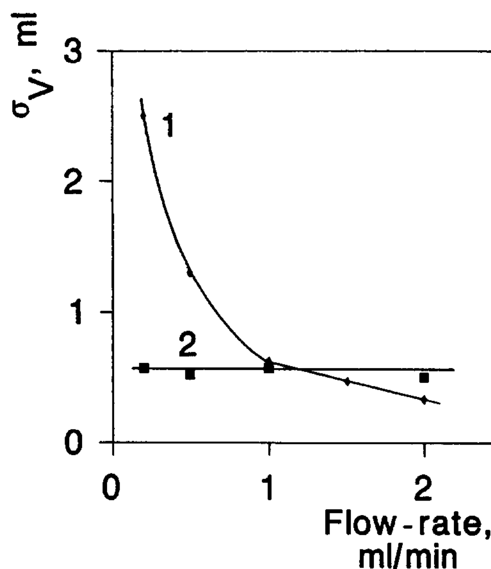

Fig. 6. Effect of the mobile phase flow-rate on band width σ_v of chicken egg albumin in anion-exchange HPMC with (1) constant gradient time and (2) constant gradient volume.

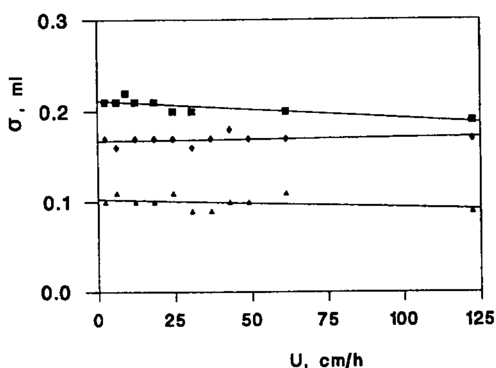

Fig. 7. Effect of flow velocity U on band width σ for various solutes. ■ = Myoglobin in 0.01 mol/l Tris–HCl buffer solution (pH 7.6); (□) acetone in water; ▲ = chicken egg albumin in 0.01 mol/l Tris–HCl buffer solution (pH 7.6) containing 0.5 mol/l NaCl.

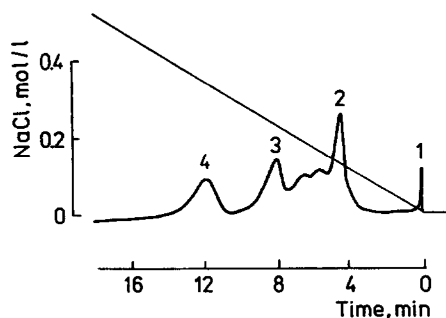

Fig. 8. HPMC separation of (1) myoglobin, (2) conalbumin, (3) chicken egg albumin and (4) soybean trypsin inhibitor using a linear mobile phase gradient. Conditions: GMA–EDMA (50:50) modified membrane containing 1 mmol/g DEA groups; mobile phase 0.01 mol/l Tris–HCl buffer solution (pH 7.6), gradient from 0 to 0.6 mol/l NaCl in the buffer, gradient time 36 min, flow-rate 5 ml/min.

rate. However, the continuity of a linear gradient is also an inherent disadvantage. The gradient runs continuously regardless of the difference between the retention times of adjacent peaks. The dead period between the peaks leads to a waste of both time and mobile phase, which should be avoided, particularly in large-scale preparative separations. Therefore, changes of the mobile phase composition in steps are more efficient provided that the concentration steps and the duration of each isocratic elution are set properly. To do that, an indicative shallow linear gradient separation has to be run to determine the composition of the mobile phase at the band maximum and the height and duration of each step are set according to these data. Fig. 8 shows the slow gradient HPMC separation of a model mixture from which the mobile phase composition at the elution of each peak was obtained and used in designing the gradient steps. The separation of individual components of the mixture with a stepwise gradient is better, overlapping of peaks is avoided and the separation time is still kept short. Fig. 9 shows an excellent separation of model protein mixtures by ion-exchange and hydrophobic interaction HPMC with a stepwise gradient.

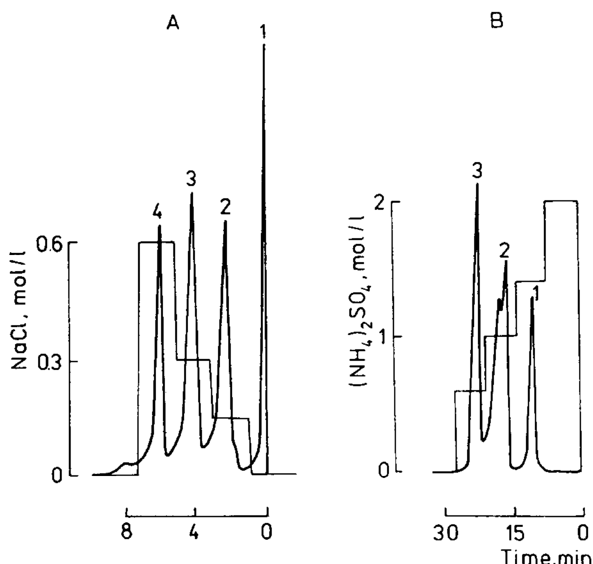

Fig. 9. HPMC separation of protein mixtures in a multiple step gradient operation. (A) GMA–EDMA (50:50) modified membrane containing 1 mmol/g DEA groups, mobile phase 0.01 mol/l Tris–HCl buffer solution (pH 7.6), gradient steps 0 (1 min), 0.15 (2 min), 0.30 (2 min) and 0.6 mol/l NaCl in the buffer (2 min), flow-rate 3 ml/min. Peaks: 1 = myoglobin; 2 = conalbumin; 3 = chicken egg albumin; 4 = soybean trypsin inhibitor. (B) Octyl methacrylate–GMA–EDMA (15:35:50) membrane, mobile phase 5 vol.% of 2-propanol in 0.02 mol/l phosphate buffer (pH 6.8), gradient steps 2, 1.4, 1.0 and 0.6 mol/l $(NH_4)_2SO_4$ in the buffer taking 6 min each, flow-rate 3 ml/min. Peaks: 1 = lysozyme; 2 = chicken egg albumin; 3 = chymotrypsinogen.

Effect of flow-rate

Fig. 10 demonstrates the dependence of the resolution factor on the linear flow velocity for

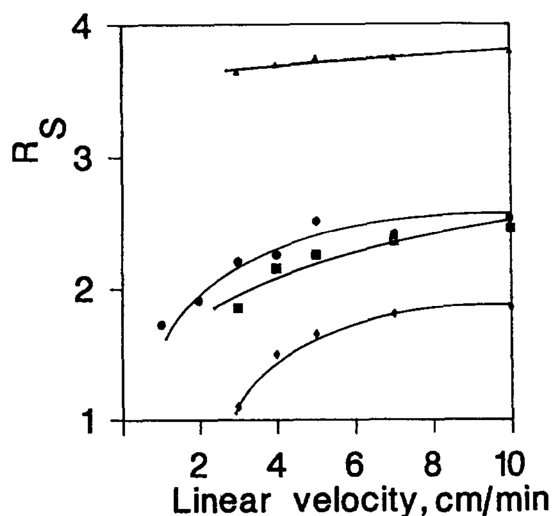

Fig. 10. Effect of the mobile phase linear velocity U on peak resolution, R_s, in HPMC. Anion-exchange separation of the pairs (■) conalbumin–chicken egg albumin, (●) chicken egg albumin–soybean trypsin inhibitor and (▲) conalbumin–soybean trypsin inhibitor and (◆) hydrophobic interaction separation of lysozyme and chymotrypsinogen A.

the pair chymotrypsinogen–lysozyme in hydrophobic interaction HPMC and for pairs formed by conalbumin, chicken egg albumin and soybean trypsin inhibitor in anion-exchange HPMC. The resolution factor increases slightly with increasing flow-rate in both modes, probably owing to narrowing of the peaks. Similar effects were also observed in the reversed-phase chromatography of tryptic digests on a column packed with poly(styrene-co-divinylbenzene) beads [36].

While the resolution changes with the linear flow velocity in all modes of HPMC, k' does so only in the ion-exchange mode and remains constant in the hydrophobic interaction mode (Fig. 11).

Enhanced diffusivity

An important characteristic of membrane chromatography is that there is almost no change in the column efficiency with increasing flow-rate (Fig. 7). Moreover, the calculated convective velocity in membrane chromatography should equal the linear flow velocity as all the mobile phase flows through the pores of the flat-body

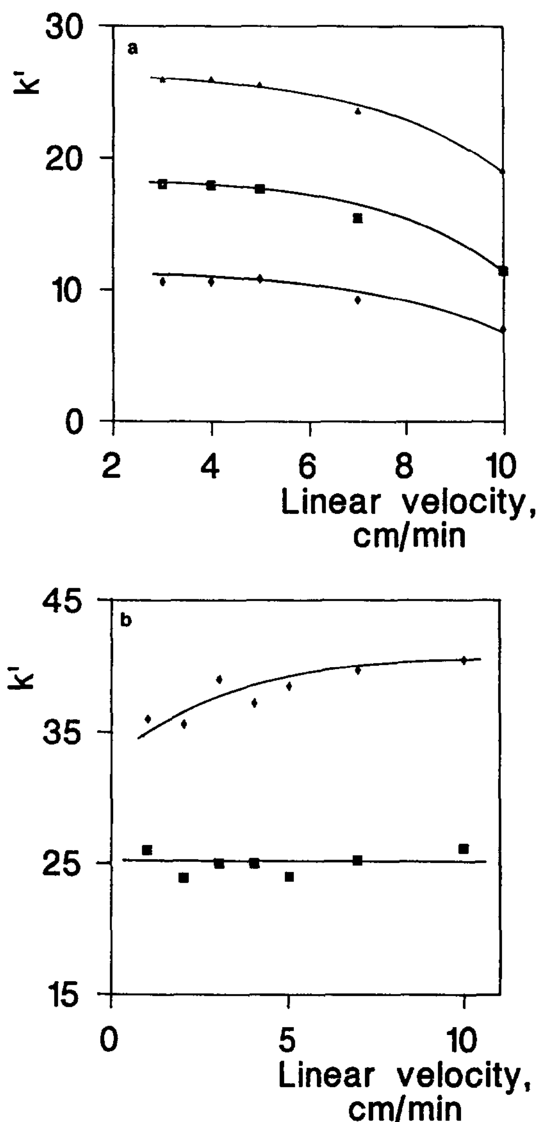

Fig. 11. Effect of the mobile phase linear velocity U on protein capacity factor k'. (a) Anion-exchange HPMC of a mixture consisting of (◆) conalbumin, (■) chicken egg albumin and (▲) soybean trypsin inhibitor; (b) hydrophobic interaction HPMC of (◆) lysozyme and (■) chymotrypsinogen A.

membrane having a slab-like geometry. The membrane permeability B_m is [37].

$$B_m = \varepsilon_m^3 d_g^2 / 150(1-\varepsilon_m)^2$$
$$= a\varepsilon_m^2 d_{pore}^2 / 150(1-\varepsilon_m)^2 \quad (6)$$

where ε_m is the membrane porosity, d_g is the diameter of the globule (microsphere) clusters within the membrane among which the transport canals are formed, d_{pore} is the pore diameter and a is defined as

$$a = d_g^2/d_{pore}^2 \quad (7)$$

Darcy's law relates the convective velocity ν_0 to the membrane permeability B_m, fluid viscosity μ and pressure drop across the membrane of thickness $l(\Delta p/l)$:

$$\nu_0 = -(B_m \Delta p/2\mu l) \quad (8)$$

Substitution of $d_{pore} = 400$ nm and $d_g = 450$ nm determined from scanning electron micrographs, and $\varepsilon_m = 0.5$ gives $B_m = 0.66 \cdot 10^{-11}$ cm^2. The intramembrane convective velocity calculated using the actual pressure drop of 5.8 MPa at a water flow-rate of 10 ml/min and a membrane thickness of 3 mm is $\nu_0 = 0.128$ cm/s. As the linear velocity calculated from the flow-rate divided by the cross-section is 0.12 cm/s, the above-expressed assumption of equivalence of the linear and intramembrane convective velocity seems to be adequate for the chromatographic membranes.

The convection of the mobile phase through a membrane also increases the diffusivity inside the pores as compared with "free" diffusion. The "apparent" or "augmented" effective diffusivity $\bar{D}_e$ is a function of both effective diffusivity D_e and Peclet number λ ($\lambda = \nu_0 l/D_e$) [37]. The Peclet number for the membrane is $1.92 \cdot 10^5$, which is high enough to permit the use of the simplified equation for the calculation of the apparent diffusivity [37]:

$$\bar{D}_e = \nu_0 l/3 \quad (9)$$

The calculation gives $\bar{D}_e = 6.4 \cdot 10^{-3}$. In contrast to the "free" effective diffusivity of proteins ($D_e \approx 10^{-7}$ cm^2/s), the diffusivity forced by the convection is more than four order of magnitude higher and the mass transfer faster. Therefore, separation in membrane chromatography is faster than in standard column methods. The dramatic increase in diffusivity in HPMC permits the use of higher flow-rates and, accordingly, with the exact gradient slope and shape, also a significant acceleration of the separation process. Moreover, the separation seems to proceed mostly in the large transport canals where convection plays the major role. It indicates that even the absence of small pores does not affect the separation properties in the HPMC membrane [38]. The concept of mass transfer enhancement by convection has already been used in heterogeneous catalysis [39] and in perfusion chromatography [40–42].

CONCLUSIONS

Detailed studies of individual effects of variable parameters in ion-exchange, hydrophobic interaction and reversed-phase protein separations confirmed that HPMC obeys the rules typical of column chromatography. The use of a stepwise gradient during the elution decreases the time necessary for separation and the amount of mobile phase used, thus decreasing the costs of the separation process. This is particularly advantageous in preparative separations. Owing to the unique absence of interparticular voids in the membrane, all the mobile phase flows through the pores of the separation medium. This results in an increase in diffusivity by several orders of magnitude augmented by the convective flow.

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