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PROTEIN SEPARATIONS ON REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY MINICOLUMNS

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SUMMARY

The resolution of cytochrome *c*, bovine serum albumin and ovalbumin on reversed-phase columns under gradient elution conditions was found to be constant or to improve as column length was decreased from 45 to 6.3 mm. The resolution remained constant even when the column length decreased to 1.6 mm. Recovery of protein in the first gradient cycle was improved by the use of short columns.

INTRODUCTION

The behavior of proteins, peptides and other macromolecules in reversed-phase high-performance liquid chromatography (RP-HPLC) columns is often much different from that of small solutes. Particularly unusual are the U-shaped plots of log k' (capacity factor) *vs.* percent organic solvent (%B) which indicate that retention is high at both large and small %B[1]. This behavior has been attributed to hydrophobic interactions at low %B and to polar interactions at high %B[1]. The rising portions of the curves can be extremely steep. A few percent change in organic solvent content can alter k' by one order of magnitude or more[1–4]. This behavior has been attributed to the large surface of a macromolecule, which can undergo multiple interactions with the stationary phase[4–6]. The multiple points of interaction require multiple solvent molecules to displace the macromolecule. Thus, log k' *vs.* %B (or log %B) plots become increasingly steep as the size and hydrophobicity of the macromolecule increases. This general behavior is predicted by various retention models[5–7].

A consequence of the great sensitivity of retention to small changes in mobile phase composition is that the resolution of macromolecules under gradient conditions is only weakly affected by column length[6,7]. As stated by Regnier[6], the columns seem to operate "on the basis of a selective desorption process", each solute being eluted at a particular %B that is largely unaffected by column length. Resolution of proteins on reversed-phase and ion-exchange columns as short as 2 cm has been shown to be nearly as large as on 25–30 cm long columns[8,9].

In this paper we have examined the resolution of proteins on reversed-phase columns as short as 1.6 mm. We have also studied the sample load capacity and recovery of protein as a function of column length.

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MATERIALS AND METHODS

Apparatus

A Model 334 gradient liquid chromatograph (Beckman, Berkeley, CA, U.S.A.) was modified by replacing the mixing chamber with a low-volume mixing chamber, designed in our laboratory. With an internal volume of 0.15 ml, gradients as sharp as 32% B/ml could be generated. Detection was performed at 280 nm by using a Beckman Model 155 spectrophotometer after removal of the heat exchanger. Data were recorded either by an Apple IIe computer (Cupertino, CA, U.S.A.) with Interactive Microware interface (State College, PA, U.S.A.) or on a strip-chart recorder. Columns of 6.3 and 45 mm length × 4.6 mm I.D. were made from a published design[10]. A 1.6 mm × 4.1 mm I.D. column was made from the ring of a Kel-F-encased frit.

Reagents

All proteins used were the purest grade available and were used without further purification. Cytochrome *c* (CYT), bovine serum albumin (BSA), ovalbumin (OVA) and ribonuclease A were obtained from Sigma (St. Louis, MO, U.S.A.). The sodium salt of insulin was obtained from Calbiochem (Berkeley, CA, U.S.A.). HPLC-grade water, HPLC-grade 2-propanol and anhydrous trifluoroacetic acid (TFA) were obtained from Fisher. LiChrospher Si 300, 10-μm particle diameter, was obtained from Rainin (Woburn, MA, U.S.A.). N-Octyldimethylchlorosilane was obtained from Petrarch (Levittown, PA, U.S.A.).

Methods

The bonded reversed-phase packing material was prepared according to a published procedure[11] by dissolving 5.00 g of *n*-octyldimethylchlorosilane in 50 g carbon tetrachloride. One gram of LiChrospher Si 300 was added, and the mixture was sonicated under vacuum until outgassing ceased. The mixture was shaken for 24 h at room temperature, then filtered through a medium-porosity glass frit, and washed three times with 30 ml carbon tetrachloride and three times with 30 ml acetone. The silica was dried at ambient temperature under vacuum for 56 h and stored in a desiccator. The silica was suspended in 2-propanol and packed into the 45-mm column at 2000 p.s.i. by using the upward-flow method[12]. The shorter columns were packed by a method developed in our laboratory in which the chromatographic tube with one end fitting at the bottom was clamped in a vertical position and connected to an aspirator vacuum. The silica slurry was added dropwise to the tube until it was filled. The vacuum was then disconnected, and the packing was leveled off with a spatula, and the other end fitting was attached. With this procedure columns of excellent efficiency could be packed in less than one minute.

Chromatographic conditions

Chromatography was performed at ambient temperature. The weak mobile phase (A) was 0.1% TFA in water. The strong mobile phase (B) was 0.1% TFA in 2-propanol. Flow and gradient rates were chosen such that a linear change in %B with time was achieved from 10 to 70%B. Confirmation of gradient shape was obtained by adding acetone to the 2-propanol eluent and monitoring the UV absor-

bance at 280 nm. Protein solutions were prepared in mobile phase A. Injection volumes were 3 and 10 μl for the 6.3 and 45 mm columns, respectively.

Resolution was calculated from retention times and baseline peak widths[12]. When the calculated value was less than 1.0, the ratio of valley height to average peak height was used to estimate resolution[12].

Protein recoveries were determined by collecting the eluted chromatographic peaks and performing Lowry protein assays[13]. Identical injections were made in the absence of a column to provide a value for 100% recovery. Fifteen μg CYT and 30 μg OVA were applied to the 6.3-mm column, while 50 μg CYT and 100 μg OVA were applied to the 45-mm column.

RESULTS AND DISCUSSION

Resolution

Fig. 1 shows a separation of five proteins on the 6.3- and 45-mm columns under conditions which yielded the highest resolution[14]: slow flow and gradient rates. Resolution was approximately the same on both columns in spite of the 7.2-fold difference in length. This is in agreement with the results of Eksteen *et al.*[9].

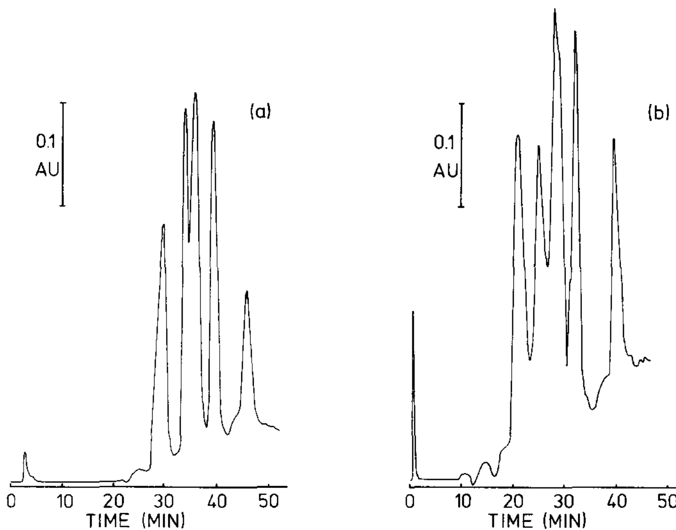

Fig. 1. Separation of five proteins on a 45-mm (a) and 6.3-mm (b) reversed-phase column. The elution order was ribonuclease A, insulin, cytochrome *c*, bovine serum albumin and ovalbumin. The amounts of each protein applied were in the ratio of 2:2:1:2:2 and totaled 50 μg. The flow-rate was 0.25 ml/min and the gradient-rate was 4%/ml (1%/min).

A more quantitative measure of the difference between columns was obtained by examining the resolution of CYT and OVA on both columns at various flow and gradient rates. The average results of at least two replicate measurements are shown in Table I. The results are surprising in that improvements in resolution of as much as 2-fold were obtained by using the shorter column. The greatest percentages of improvement were seen when the flow-rate was low and the volumetric gradient rate

TABLE I

EWAOLUTION OF CYTOCHROME *c* AND OVALBUMIN

		Flow-rate (ml/min)			
		0.25	*0.5*	*1.0*	*2.0*
6.3-mm column					
Gradient-rate	32	1.8	1.5	1.0	1.0
	16	2.7	2.0	1.2	1.1
(Δ%B/ml)	8	3.3	2.4	1.5	1.1
	4	5.1	3.4	2.4	1.8
45-mm column					
Gradient-rate	32	1.0	0.9	0.9	0.7
	16	1.2	0.9	0.9	0.9
(Δ%B/ml)	8	1.9	1.7	1.2	1.2
	4	4.4	2.0	1.5	1.4

(Δ%B/ml) was large. The uncertainty in these measurements was estimated to be ±0.3.

To explain the results qualitatively, one can imagine an extreme case in which the solute goes from strong retention to no retention over an infinitely narrow range of %B. If the solute is initially adsorbed at the top of the column, migration will not begin until the %B has reached a certain critical value, and then the solute will migrate like a non-retained solute. In this case, the peak width would increase with the square root of column length (L) and resolution (R_s) would be inversely proportional to $\sqrt{L}$. In addition, different solutes would always be eluted at certain points in the gradient. Actually, this case is one extreme of the more general gradient elution theory of Snyder[7,15].

Snyder's gradient elution theory, when applied to macromolecules, predicts that typical gradient elution conditions will yield average capacity factors ($\bar{k}$) close to zero[7], as described above. In addition, he predicts that the mobile phase composition at elution will be almost independent of the column surface area and, thus, that the column itself will appear to be unimportant[7]. He does not, however, go so far as to predict better resolution on shorter columns. Nevertheless, consider Snyder's equation for resolution under gradient conditions[15]

$$R_s = \frac{\sqrt{N}}{4}(\alpha - 1)\frac{1}{1 + 1.15b} \quad (1)$$

where N is number of theoretical plates and α is the selectivity factor. The gradient steepness parameter, b, is given by

$$b = \frac{V_m \varphi}{F} \log \frac{k'_A}{k'_B} \quad (2)$$

where V_m is the column void volume (ml), F is the flow-rate (ml/min), φ is the gradient rate (volume fraction change in B/min) and k'_A and k'_B are the isocratic capacity factors of the earlier-eluted solute in pure solvent A and B, respectively[15]. Note that 100% φ/F is the gradient rate in Δ%B/ml, as given in Table I.

For protein separations, $\log k'_A/k'_B$ is frequently very large[2,9] (steep slope of the plot of $\log k'$ *vs.* φ) so that $1.15b \gg 1$. Then

$$R_s = \sqrt{N}\,(\alpha - 1)\, F\Bigg/\left(4.6 V_m \varphi \log \frac{k'_A}{k'_B}\right) \qquad (3)$$

Since the column void volume, V_m, increases with L while $\sqrt{N}$ increases with $\sqrt{L}$, the overall effect of the N and V_m terms is that resolution is proportional to $1/\sqrt{L}$, *i.e.* resolution decreases with increasing column length. This is the same effect as that qualitatively predicted earlier.

From isocratic data[2] on the elution of cytochrome *c*, we estimate the value of b to be in the range 1–6 for our shorter column and 4–30 for our longer column. Thus, we would expect eqn. 3 to govern most of our separations. We would predict a 2.7-fold improvement in resolution on the shorter column, the observed average being 1.5.

The effects of flow-rate and gradient rate on resolution are also indicated by the data in Table I. Across each row of the table, φ/F is constant so that F only affects the number of plates. Under the conditions normally used in HPLC, N is approximately proportional to $1/F$, so that the 8-fold increase in F across each row should lead to a 2.8-fold decrease in resolution[12]. The measured decrease of 2.5-fold on the 6.3-mm column and 1.9-fold on the 45-mm column are in reasonable agreement with the prediction.

Each column in Table I represents an 8-fold change in the gradient rate. According to eqn. 3, an 8-fold increase in gradient rate should produce of an 8-fold decrease in resolution. The data indicate an average decrease of 2.3-fold for the shorter column and 2.6-fold for the longer column. Similar effects of gradient and flow-rate have been observed by others[14,16,17].

It therefore appears that our data are in at least qualitative agreement with eqn. 3. However, we are hesitant to accept this explanation for several reasons. Firstly, the b values of longer columns would generally be at least as large as those of our columns, so we would have expected significant changes in resolution in the chromatograms of other workers[9,11] when column length was varied. Such changes were not observed.

Secondly, we have more closely examined our data to determine whether the source of the improved resolution was a peak width change, as predicted by our qualitative argument, or shifts in the peak positions. In some cases we found that the peak widths were narrower on the shorter column, but in other cases the peaks were spread farther apart. Fig. 2 shows an example in which the peak positions shifted significantly, while Fig. 3 shows an example where some peak shifts occurred and where the OVA peak was significantly narrowed. Some shifting is also apparent in Fig. 1. The shifting and narrowing showed no discernible trend with regard to

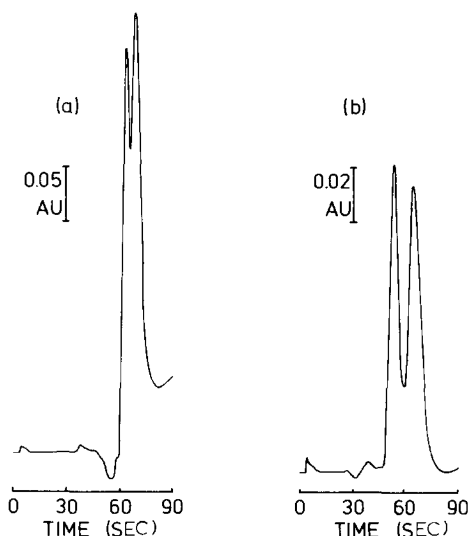

Fig. 2. Resolution of cytochrome *c* and ovalbumin on a 45-mm (a) and 6.3-mm (b) column at a flow-rate of 2 ml/min and a gradient-rate of 32%/ml (64%/min). 50 μg CYT and 100 μg OVA were applied in (a) while 15 μg CTC and 30 μg OVA were applied in (b).

gradient-rate or flow-rate. In other column length studies the difference in peak retention times have generally been constant[7,9], although the chromatograms of Eksteen *et al.*[9] show small shifts in the position of some peaks on 2-cm *vs.* 25-cm columns. We also measured the resolution of BSA–OVA and found that the resolution was nearly the same or slightly better on the shorter column. A tendency for the BSA peak to split into multiple sharp peaks (polypeaks[3]) was observed at intermediate gradient rates.

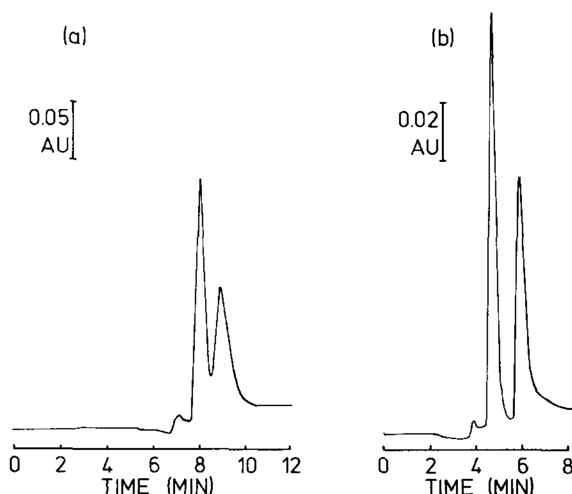

Fig. 3. Resolution of cytochrome *c* and ovalbumin on a 45-mm (a) and 6.3-mm (b) column at a flow-rate of 0.25 ml/min and a gradient-rate of 32%/ml (8%/min). Other conditions were as in Fig. 2.

These observations indicate that the resolution differences cannot be explained in the simple manner suggested by eqn. 3. They could be caused by other factors, such as the curvature of the log k' *vs.* φ plots or the steeper slope of this plot for OVA compared to CYT or BSA. These factors violate the assumptions made in deriving eqn. 1[15]. The resolution changes may be due to such factors as solvent demixing or imperfections in the gradient which might affect the two columns differently. It might well be that, if we had measured the resolution of several more protein pairs, we would have found some where resolution declined as the column length decreased. We therefore believe that with our limited data we can only conclude that resolution remains approximately constant as column length decreases. We also separated a mixture of ribonuclease A, CYT, and OVA on the 45- and 6.3-mm columns and on a column only 1.6 mm long (Fig. 4). In spite of the fact that this latter column was grossly overloaded (note the protein eluted in the void volume) and poor geometry (2.6 times wider than it was long), the resolution was virtually identical to that for the other two columns. Even if it were not overloaded, the number of theoretical plates in this column would have been less than 10 (based on size-exclusion chromatographic data). It is clear that the stationary phase plays only a minor role in this type of separation.

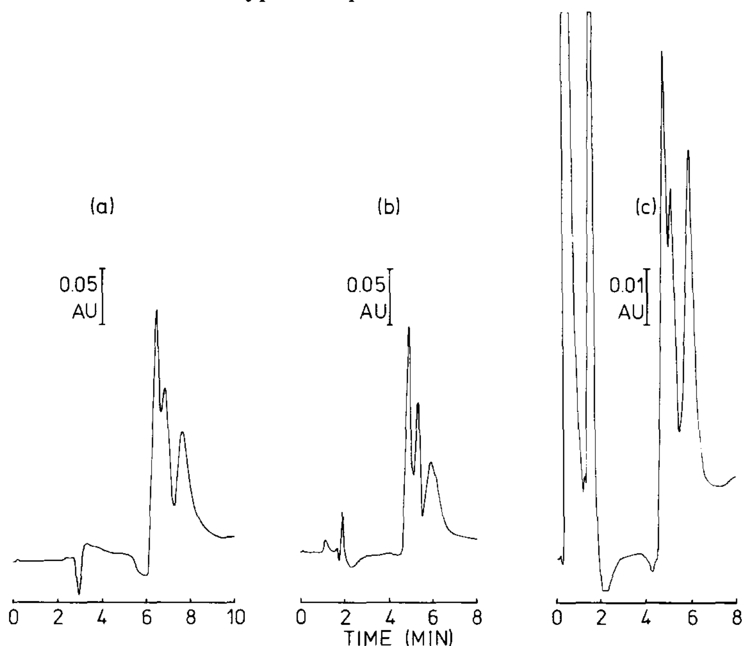

Fig. 4. Resolution of ribonuclease A, cytochrome *c*, and ovalbumin on 45-mm (a), 6.3-mm (b) and 1.6-mm (c) columns. The flow-rate was held at 0.1 ml/min for 1 min after injection, then increased to 0.5 ml/min. A 32%/ml (16%/min) gradient was begun at 2 min. 73 μg of protein in a ratio of 5:1:5 were applied to all columns.

Sample capacities

It is well know that maximum sample load tends to decrease with decreasing column length[11]. One measure of the sample capacity is the total adsorption capacity under flow conditions which we have called the "dynamic capacity"[18].

The dynamic capacities for the 6.3- and 45-mm columns were found by using single injections of increasing amounts of OVA until a peak was eluted at the void volume. The columns were washed several times between injections to clear them of retained proteins. The values obtained are listed in Table II. The capacity of the 45-mm column was several milligrams, in agreement with the results of others[11]. The capacity of the 6.3-mm column was much lower, but still well above the minimum needed for quantitative analysis. The capacity per unit length was also lower for the shorter column, presumably due to incomplete radial distribution of the sample at the top of the column[11].

TABLE II

DYNAMIC CAPACITIES FOR OVALBUMIN

Flow-rate: 0.25 ml/min; gradient-rate: 4%/ml (1%/min).

Column length (mm)	*Capacity (μg)*	*Capacity/length (μg/mm)*
6.3	200	32
45	2500	55

Recoveries

The recoveries of proteins, especially hydrophobic proteins, are sometimes poor in RP-HPLC[11]. One might expect that a smaller column would exhibit better recoveries because of its lower surface area. To test this, we measured the total recoveries of CYT and OVA from the 6.3- and 45-mm columns by collecting the effluent from several gradient cycles. The total recoveries ranged from 81 to 97% and did not show a significant column length effect. CYT was recovered in better yield from the shorter column and OVA was recovered in better yield from the longer column.

The shorter column did exhibit a lesser tendency toward "ghost peaks". As shown in Fig. 5, with the shorter column more OVA was recovered in the first gradient cycle and significantly less was recovered in the second and third cycles.

CONCLUSION

The advantages of very short reversed-phase HPLC columns for macromolecule separations include lower cost and better recovery in the first gradient cycle. Resolution may improve somewhat or remain approximately constant. Separation time may decrease somewhat due to the smaller void volume, but under the slow gradient rates, which give the best resolution, this difference becomes negligible. The disadvantage is lower sample capacity which, even if resolution increases, eventually causes detectability problems.

Since the band broadening under the conditions employed here is likely to be primarily due to slow mass transfer between stagnant and moving mobile phase, the greatest potential for these short columns may be in the use of very small diameter particles. We estimate[12,19] that a 2-mm long column, operated at a linear velocity of 0.1 cm/sec with a particle diameter of 0.2 μm would operate at a pressure of about

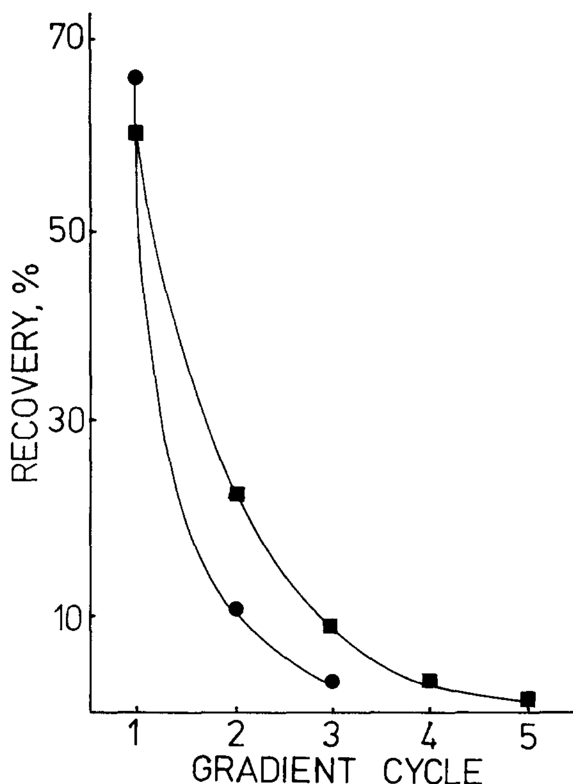

Fig. 5. Recovery of ovalbumin in successive gradient cycles on a 45-mm (■) and 6.3-mm (●) column. The amount of OVA applied was 100 and 30 μg, respectively. The flow-rate was 0.5 ml/min and the gradient-rate was 32%/ml (16%/min).

3000 p.s.i. Compared to a similar column containing 10-μm particles, this column should yield better resolution by at least a factor of 15 due to both the smaller particle diameter and operation near the optimum reduced velocity.

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