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MICROBORE FLOW-RATES AND PROTEIN CHROMATOGRAPHY

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SUMMARY

Reversed-phase chromatography of proteins on microbore columns can achieve sensitivities that exceed those for standard-bore columns by a factor of 10–20, when operated at the same linear velocities. These gains in sensitivity are accompanied by proportional reductions in peak volume. Sensitivities on standard- (4.6 mm I.D.) and narrow-bore (2.1 mm I.D.) columns have been further improved by reducing the flow-rates to those typical for microbore (1 mm I.D.) columns. We have investigated the role of flow-rate in determining peak volumes for a constant time gradient and found that flow-rate affects peak volume to a much greater extent than column diameter. Column length was not found to have a significant effect on either peak volume or sensitivity. We have found that a four-fold reduction in flow-rate results in at least a two-fold reduction in peak volume over the flow-rate range from 25 to 400 μl/min. Recovery of proteins in smaller volumes should prove beneficial to subsequent protein characterization methodologies.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) has become a vital method for protein isolation and characterization. Most parameters of this technique have been well characterized and practical guidelines have been established. Both acetonitrile and propanol have proven useful solvents in gradient elution[1,2], and trifluoroacetic acid[3] an important mobile phase modifier. Wide-pore packings (30 nm or larger) produce superior results[4], and short columns often improve the recovery[5,6]. The effects of alkyl chain length (from methyl to octadecyl) on such bonded phases, are more subtle[7], as is the question of ligand density[8]. But in general, comparable results can be obtained on several wide-pore materials having either octyl or octadecyl bonded phases[9].

The effects of flow-rate have received far less attention. Flow-rate has too often been examined in conjunction with other parameters such as gradient slope[10], minimizing the importance of flow. Although there may be a sound theoretical basis for such an approach, it obscures practical relationships between flow-rate and both resolution and sensitivity. Little attention is usually given to selecting flow-rates; 1.00, 0.20, and 0.05 ml/min are automatically selected for 4-, 2-, and 1-mm I.D. columns. There have been reports suggesting that either faster or slower rates are more ap-

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propriate[11,12]. The objective of this study is to clarify this issue with particular emphasis on peak volume effects.

Because volume reduction by lyophylization is often accompanied by protein loss[13], it should be avoided when one attempts to characterize trace amounts of proteins. If microsequencing is an objective, then proteins should be collected in small volumes (30–100 μl) that are compatible with this instrumentation. Microbore chromatography with 1-mm I.D. columns is one approach to achieving such peak volumes[14]. A major objective of this study was to determine whether wider I.D. columns can also be used to generate such peak volumes. An attempt was also made to shed additional light on whether proteins behave chromatographically like a special class of small molecules[15] or like the models of Regnier[16] and Jennissen[17], which suggest that proteins stick very tightly to the packing with little or no partitioning until the mobile phase can adequately solvate and desorb the protein.

MATERIALS AND METHODS

Equipment

Chromatography was conducted with a Model 130 separations systems (Applied Biosystems, Foster City, CA, U.S.A.), equipped with a 2.4-μl flow cell. The system dead-volume includes a small static mixer (*ca.* 80 μl) and a micro-dynamic mixer (*ca.* 220 μl) for a total of 300 μl. All columns were packed with RP-300, a C_8 bonded phase on a 30-nm pore, 7-μm support from Brownlee Labs. (Santa Clara, CA, U.S.A.). Columns of 4.6 and 2.1 mm I.D. were obtained from Brownlee Labs. in cartridges, whereas the 1-mm diameters were in a standard column format.

Chemicals and standards

Acetonitrile, sequencer grade, and trifluoroacetic acid (TFA), peptide synthesis grade, were obtained from Applied Biosystems. The protein standard contained insulin, cytochrome *c*, α-lactalbumin, carbonic anhydrase, and ovalbumin, as a lyophilized powder (Applied Biosystems). The standard was diluted to a concentration of 25 ng/μl with 0.1% TFA and 100-μl aliquots were kept frozen in Eppendorf tubes. The tubes were thawed on the day of use. The tryptic peptide digest of apomyoglobin (Applied Biosystems) was also prepared in 0.1% TFA at a level of 20 pmol/μl and used directly.

Chromatographic procedures

The solvent system for gradient elution consisted of: solvent A, 0.1% TFA and solvent B, acetonitrile–water (70:30) containing 0.08% TFA. Three slightly different linear gradients were employed. One gradient began at 30% B and reached 90% B in 25 min. Another began at 35% B and ramped at 2%/min till 85% B and reached 95% B 10 min later. This was held until ovalbumin was eluted. For the separation of tryptic peptides from apomyoglobin, the gradient started at 5% B and incremented linearly to 20% B in 10 min, followed by an increase to 80% B in 25 min. The column temperature was 35°C throughout and detection was routinely at 214 nm with a time constant of less than 1 s.

The variance of the LC system (extra-column) reflects peak broadening due to mixing volumes in the system. To obtain good column performance this variance

should be less than 10% of the peak variance in an ideal system. The variance of the HPLC system was determined to be about 12 μl^2 by the direct injection technique[18]. Most peak variances were greater than 100 μl^2, *i.e.* peak volumes of 40 μl or more. Peak volumes below 25 μl were corrected for this extra-column variance.

RESULTS AND DISCUSSION

Elution at microbore flow-rates is not considered feasible for standard columns, but the chromatograms in Fig. 1 demonstrate that proteins can be efficiently eluted from a 220 × 4.6 mm I.D. column in less than 1 h at flow-rates as low as 100 μl/min. The comparable peak spacing for each of the three flow-rates indicates that the gradient time-profile controls the elution. In this example protein elution is governed primarily by the time when the gradient reaches a certain percentage of acetonitrile. This behavior is explained by the theory suggesting that proteins stick very tightly to the packing and once desorbed, have very little further interaction with the bonded phase.

The total elution volume in the three chromatograms varies from 2 ml, the approximate column void-volume, to 8 ml at the higher flow-rate. Because there is

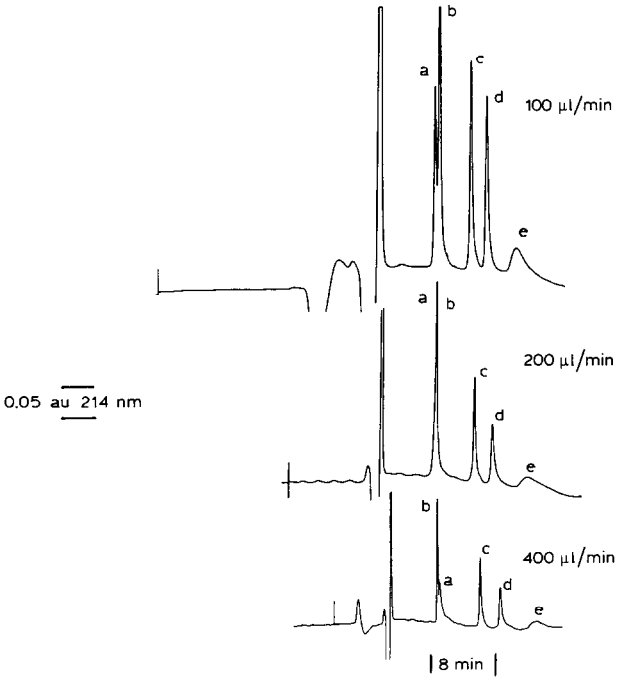

Fig. 1. Protein separations on a standard column at low flow-rates. Five proteins (a, insulin; b, cytochrome *c*; c, α-lactalbumin; d, carbonic anhydrase; and e, ovalbumin) were separated on a 220 × 4.6 mm I.D. column, Aquapore RP-300, eluted with TFA (solvent A) and acetonitrile–water (70:30) containing 0.8% TFA (solvent B). A 50-μl aliquot, containing 1.25 μg of each protein, was injected. After injection the gradient was increased at a rate of 2.4%/min in solvent B starting at 30% B. Flow-rates are as given. Detection was at 214 nm (0.5 a.u.f.s.).

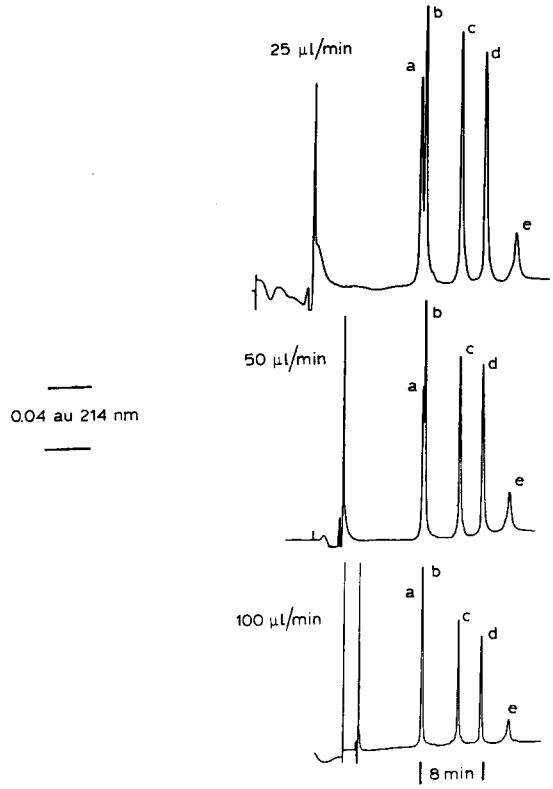

Fig. 2. Protein separations on a microbore column. The same proteins as in Fig. 1 were injected on a 250 × 1.0 mm I.D. Aquapore RP-300 column. Gradient conditions were as in Fig. 1, except flow-rates were 25, 50, and 100 µl. Only 125 ng (5 µl) of each protein was injected. Detection was at 214 nm (0.4 a.u.f.s.), and peak identification was the same as in Fig. 1.

little change in the time distribution of the peaks, the peaks must be more diluted at the higher flow-rate. Peak concentrations are in fact three times lower at 400 compared to 100 µl/min, as observed from peak heights. This difference is not four-fold because the peak widths are slightly wider at the slower flow-rate, so there is a slight penalty in lower resolution for the added benefit of higher concentrations and smaller volumes.

Ovalbumin recovery is poor under these conditions of slower than normal flow-rate. Poor ovalbumin recovery at low flow-rates has also been observed on 4.6 mm I.D. columns by O'Hare *et al.*[7]. Surprisingly, resolution improved for insulin and cytochrome *c* when the flow-rate was decreased. Although not immediately apparent, this improvement stems from a change in selectivity as insulin moves in front of cytochrome *c*. Similar results at lower flow-rates have been reported by Glajch *et al.*[19]. The observed change in selectivity probably reflects different mobilities for small differences in acetonitrile concentration that result under the different flow-rate conditions.

Reduced flow produces similar effects on 1-mm I.D. columns. A comparable

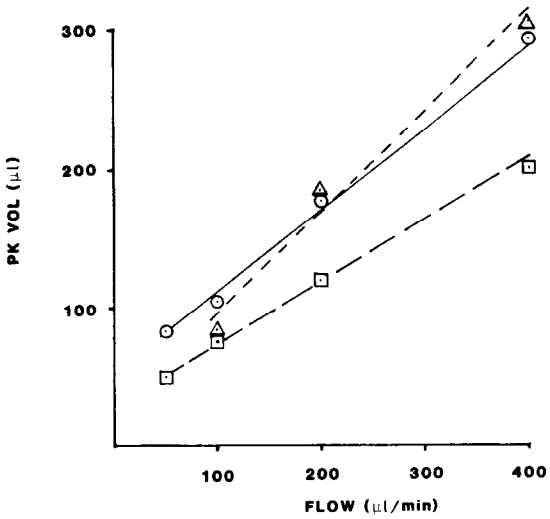

Fig. 3. Flow-rate dependence of peak volumes on standard-bore columns of varying lengths. The protein standard (50 µl) was chromatographed on three lengths [(△) 220, (□) 100 and (○) 30 mm] of Aquapore RP-300, all 4.6 mm I.D. The previously described solvent system was used but the gradient began at 35% solvent B and increased at 2%/min until all the proteins eluted. Flow-rates were varied from 50 to 400 µl/min.

series of chromatograms in Fig. 2 illustrates that peak volumes decrease with flow-rate, so that volumes at 25 µl/min are about half of those at 100 µl/min. Again cytochrome *c* emerged earlier relative to insulin at the lower flow-rates. The recovery of ovalbumin is substantially improved on the 1-mm I.D. column compared to the

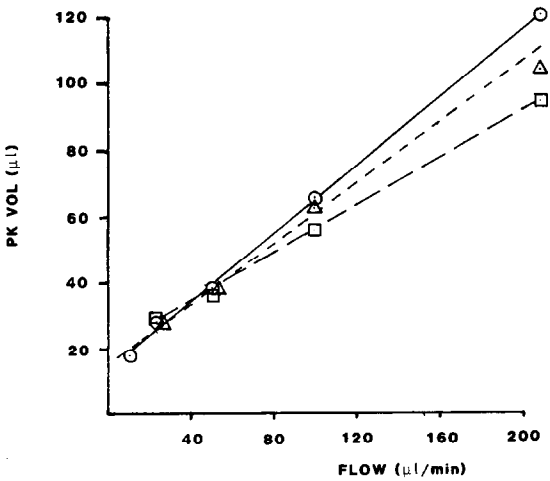

Fig. 4. Flow-rate dependence of peak volumes on narrow-bore columns of varying lengths. The protein standard (5 µl) was chromatographed on three lengths [(△) 220, (□) 100 and (○) 30 mm] of Aquapore RP-300, 2.1 mm I.D. using the same conditions as described in Fig. 3. Flow-rates were varied from 20 to 200 µl/min.

previous series of chromatograms. Pearson[6] reported that ovalbumin recovery improved with shorter columns. Perhaps, simple reduction in the packing volume may explain the observed improvement on the smaller I.D. columns.

This same series of chromatograms was repeated on a 2.1-mm I.D. column. Peak height comparisons between columns indicated that increases in height are proportion to the square of the diameter ratio (larger–smaller) at constant linear velocity, but proportional to the simple ratio at constant volume flow. This data fits with the desorption model if the time required for desorption is the key determinant to peak concentration. Thus higher flow-rates tend to merely dilute the protein band rather than speed the band elution rate.

The overall relationship between flow-rate and peak volume can be gleaned from the graphs in Figs. 3–5. The data in Fig. 3 illustrate a linear relationship between peak volume and flow-rate for three lengths of 4.6-mm I.D. columns. Each point represents the average peak volume for α-lactalbumin and carbonic anhydrase, which were baseline resolved. Although there are some differences in column efficiencies, a four-fold increase in flow-rate will produce about a two-fold increase in peak volume on the 4.6 mm-I.D. columns. Protein can be collected in less than 100 μl on these columns, but the flow-rate must be reduced to less than 100 μl/min. The 100-mm long column had the highest efficiency and produced the smallest peak volumes at any given flow-rate.

The same general trend is observed for 2-mm I.D. columns in Fig. 4. An increase in flow-rate from 50 to 200 μl/min approximately doubled peak volumes. To achieve peak volumes of less than 100 μl on the 2-mm columns, the flow-rate needs only to be 200 μl/min, which is a typical rate. Because the glass fiber filters used in

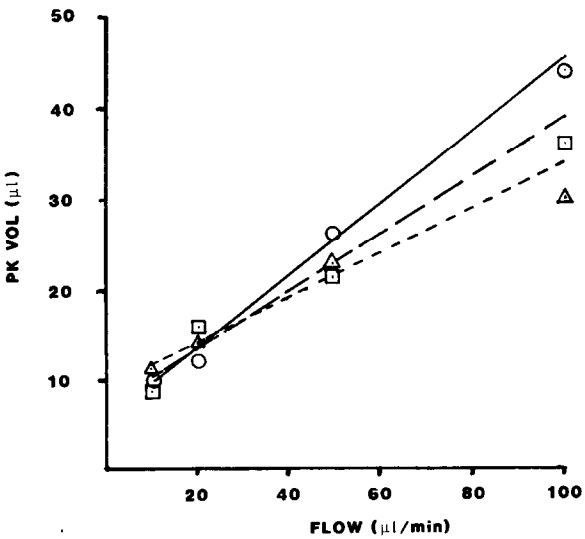

Fig. 5. Flow-rate dependence of protein peak volumes on microbore columns of varying lengths. The protein standard (5 μl) was chromatographed on three lengths [(△) 250, (□) 100 and (○) 50 mm] of Aquapore RP-300, 1.0 mm I.D. using the same conditions as described in Fig. 3. Flow-rates were varied from 10 to 100 μl/min.

gas-phase sequencing can only retain about 30 μl of fluid, flow-rates of 40 μl/min would be required to produce peak volumes appropriate for filter disks in sequencing. The closeness of the lines indicates that the 2-mm I.D. column efficiencies are more nearly equal in this study.

The same trend is observed for 1-mm I.D. columns (Fig. 5), except that peak volumes are even lower for every flow-rate. To achieve 30-μl peak volumes on any of the 1-mm I.D. columns, only requires a flow-rate of 60 μl/min. Very small protein peak volumes are achievable with the 1-mm I.D. reversed-phase columns. Peak volumes as low as 10 μl are achievable with gradient elution at a flow of 10 μl/min, which is the lower limit of gradient capability of the instrumentation used in this study. Again the closeness of the three plots indicates that the three columns were packed at about the same efficiency. Although this instrumentation has very low, extra-column variance, the peak data for flows below 40 μl/min had to be corrected for the bandbroadening caused by the system.

Comparing the three graphs at a common flow-rate of 100 μl/min reveals that the average peak volumes (for the three column lengths) are approximately 90, 60, and 30 μl for the 4.6-, 2.1-, and 1.0-mm I.D. columns, respectively. Reciprocally, the sensitivity of the smallest diameter is three-times greater than the widest diameter

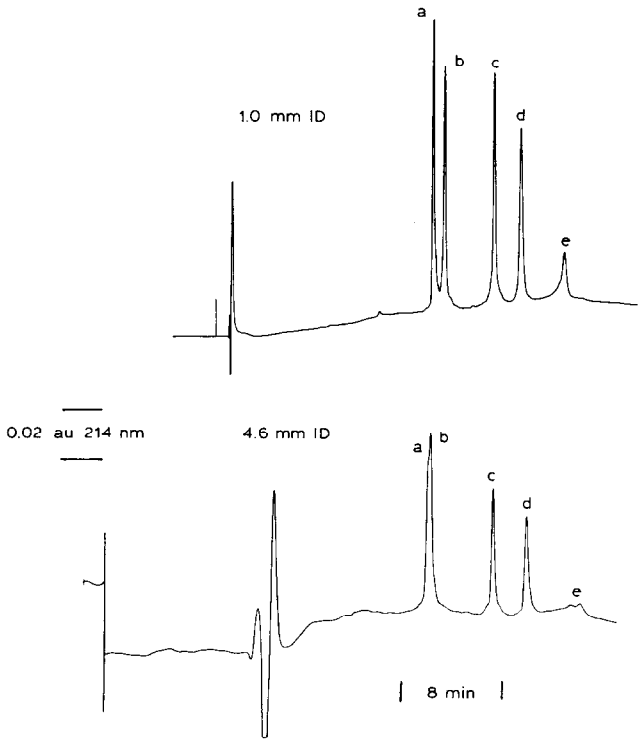

Fig. 6. Effect of column diameter on protein separations at low flow-rates. The protein standard (5 μl) was injected onto both a standard-bore, 220 × 4.6 mm I.D. Aquapore RP-300 column, and a microbore, 250 × 1.0 mm I.D. Aquapore RP-300 column. The gradient conditions were the same as in Fig. 3, and the flow-rate was 100 μl/min for both. Peak identifications as in Fig. 1.

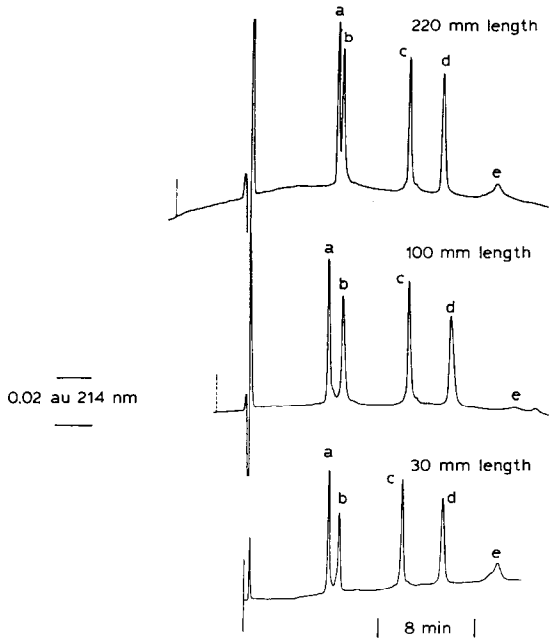

Fig. 7. Effect of column length on protein separations at low flow-rates. Three lengths (220, 100 and 30 mm) of narrow-bore (2.1 mm I.D.) columns, Aquapore RP-300 were used under the chromatographic conditions described in Fig. 3 with a flow-rate of 100 μl/min. Peak identifications as in Fig. 1.

column. Typically, the sensitivity difference would be expected to be in the range of 10–20 in favor of the 1.0-mm I.D. column, when the latter is operated at a flow-rate of 50 μl/min and the 4.6-mm I.D. column at 1000 μl/min[14].

The protein separations in Fig. 6 illustrate the effect of column diameter on sensitivity when flow-rate and gradient-time program are held constant. The sensitivity enhancement is only about two-fold, but detectability is even higher on the 1-mm column because the baseline noise is greater for the 4.6-mm column. This higher noise probably results from inadequate solvent mixing at such very low linear velocities. The linear velocity on the wider bore column is 25 times lower. Resolution and ovalbumin recovery are also superior on the 1-mm column.

Column length has oft been reported to play a negligible role in protein chromatography[5,6]. This observation is reaffirmed by the series of chromatograms in Fig. 7. Shorter columns are expected to produce less peak dilution. Theory suggests that peak sensitivity to be inversely proportional to the square root of the column length. However, a seven-fold reduction in column length has little effect on protein detection. In fact, peak heights are somewhat higher, reflecting either better efficiency or a small amount of random variation. The decrease in resolution for insulin and cytochrome *c* on the longest column is probably a result of different organic solvent concentrations at the column exit, which produced poorer selectivity.

Because peptides often exhibit chromatographic behavior similar to that for proteins, the effect of reduced flow-rates on peptide chromatography was examined. As in the case of proteins, similar sensitivity gains were observed for peptides at reduced flow-rates (Fig. 8). The tryptic digest chromatograms are typical of the re-

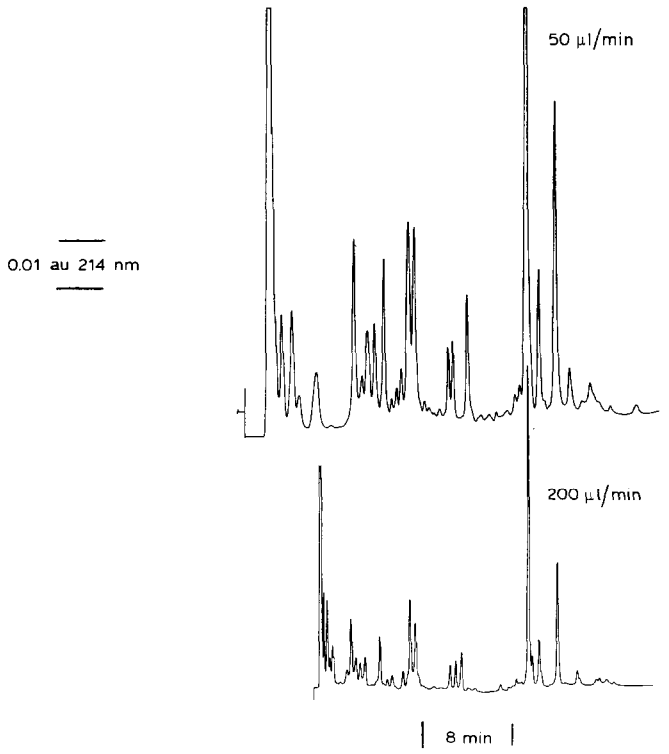

Fig. 8. Tryptic peptide separations on a short column at low flow-rates. Tryptic digest of apomyoglobin (100 pmol in 5 µl) was chromatographed on a 30 × 2.1 mm I.D. Aquapore RP-300 column using the same solvent system as in Fig. 1. The gradient began at 5% solvent B as described under *Chromatographic procedures*. Flow-rates were 50 and 200 µl/min with detection at 214 nm (0.1 a.u.f.s.).

sults observed for the limited number of peptide samples which we have studied. The separations were conducted on a minicolumn, 30 × 2.1 mm I.D., and show good resolution of this complex mixture with a peak capacity of about 100 for a 45-min gradient. The first two or three peptides to be eluted do not show an appreciable increase in sensitivity. These more hydrophilic molecules may behave more like small molecules than the other more hydrophobic peptides. Some work on protein elution at high initial organic solvent levels also suggests that early-eluted proteins may behave more like small molecules, at least with regard to flow-rate effects.

CONCLUSIONS

We have found that flow-rate reduction is a simple technique for decreasing protein peak volumes in gradient elution on reversed-phase packings. In particular, peak volumes vary proportionally with flow-rate when the gradient program is held constant. A four-fold reduction in flow-rate results in about a two-fold reduction in peak volume for a 2%/min gradient on a 7-µm packing. Length does not appear to be a significant factor in the efficiency of protein peaks on reversed-phase columns. Hence, it has little effect on peak volumes or sensitivities in protein chromatography.

Column diameter, however, does affect peak volumes and sensitivities. By selecting smaller column diameters and using lower flow-rates, peak volumes can be substantially reduced. On 2 mm-I.D. volumes, peak volumes of 30 µl or less are achievable by gradient elution at 40 µl/min flow-rates. Reducing peak volumes to 30 µl or less is particularly useful because it allows direct peak collection on glass fiber discs for sequencing.

Even conventional columns (4.6 mm I.D.), benefit from reduced flow-rates. At 100 µl/min flowrates, peak volumes were reduced to about 90 µl. Although slower flow-rates are possible, peak distortion and severe baseline upsets were observed at flow-rates below 50 µl/min. At such low linear velocities, recovery of ovalbumin was very poor, suggesting that hydrophobic proteins may not chromatograph well under such conditions.

Under the reported gradient conditions the column diameter has a proportional effect on peak volume, whereas the square of the diameter ratio is typical for small molecules. Because linear velocities are much higher on the 1-mm columns, classical theory could still explain this discrepancy by pointing to worse mass transfer on the 1-mm I.D. column. However, these results are also consistent with another model for protein chromatography.

The model proposed by Geng and Regnier[20] emphasizes that proteins are adsorbed on the stationary phase through multiple interactions, and are desorbed over a narrow range of organic solvent concentrations, having little further interaction with the stationary phase during elution. Our observations indicate that the mobile phase volume containing the required concentration of organic solvent to effect desorption plays a substantial role in determining peak volumes.

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