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MICROBORE REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS WITH CONVENTIONAL GRADIENT EQUIPMENT FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Reversed-phase high-performance liquid chromatography with microbore columns (50 × 1.0 mm) was used effectively for the separation and analysis of proteins down to 1 ng at flow-rates of 0.1–0.2 ml/min. With the use of standard low-pressure gradient HPLC equipment, the peak volumes were five times smaller when compared with a conventional column at equal chromatographic efficiencies and analysis time. The sensitivity of detection was further increased by a reduction in solvent peaks, resulting in a 20-fold overall increase.

INTRODUCTION

High-performance liquid chromatography (HPLC) has become a generally applied tool for the separation and purification of proteins from complex mixtures[1]. Relatively large volumes of a dilute protein solution can be separated and concentrated with gradient elution on ion-exchange or reversed-phase columns.

With the commonly used UV detection, the lowest amount of protein which can be detected on analytical columns (4.6 mm I.D.) is of the order of 20–50 ng. Besides the low protein concentration during elution, the sensitivity is limited by baseline disturbances caused by solvent peaks. For analytical applications the detection sensitivity can be increased by using lower flow-rates, but in order to maintain separation efficiency the gradient time has to be increased concomitantly[2], which results in much longer analysis times. Apart from the practical discomfort, this may negatively influence the recovery of some proteins[3].

Therefore alternative methods are required to obtain smaller peak volumes and a higher detection sensitivity. One way to reach this goal might be through the use of microbore columns (1.0 mm I.D. or less). In isocratic elution, the bandwidth and thus the peak volume decreases (and sensitivity increases) with the square of the column diameter and directly with column length if the number of theoretical plates remains the same[4]. The plate number can hardly be expected to be the same for two columns of different sizes, and the observed bandwidths in gradient elution are also

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dependent on the gradient time. A recently described model for the separation of proteins in reversed-phase HPLC gradient elution predicts that the peak volume and resolution are more complexly related to column dimensions than in isocratic elution[5].

As reports on the application of microbore columns for protein separation are scarce[6] further research is needed on this subject. In the present study it will be shown that, by using microbore columns with otherwise conventional gradient HPLC equipment, much smaller peak volumes and thus higher sensitivity of detection can be obtained, without loss of resolution or speed when compared with a conventional analytical column. It was also found that, apart from peak volume reduction, the sensitivity was further improved indirectly through a reduction in the solvent peaks at the lower flow-rate.

EXPERIMENTAL

Reagents and materials

Bovine pancreatic ribonuclease and bovine erythrocyte carbonic anhydrase were from Sigma (St. Louis, MO, U.S.A.), horse heart cytochrome *c* from Boehringer (Mannheim, F.R.G.) and ovalbumin from Millipore (Freehold, NJ, U.S.A.). Acetonitrile (HPLC/Spectro grade) was obtained from Alltech (Deerfield, IL, U.S.A.) and trifluoroacetic acid (TFA, Uvasol) from E. Merck (Darmstadt, F.R.G.).

Chromatography

Chromatography was performed with a system consisting of an LKB 2150 pump, a Rheodyne 7125 injector equipped with a 10-μl loop and a Waters 441 detector. Both flow cells (volumes 14 and 1.9 μl) employed had an optical path length of 10 mm. Low-pressure gradients were generated by an Atom microcomputer (Acorn Computers, Cambridge, U.K.), interfaced with an LFYX three-way solenoid valve (Lee, Westbrook, CT, U.S.A.)[7].

Test solutions of the protein standards (25–50 μg/ml), prepared in triply distilled water, were eluted with linear gradients of solvent B in solvent A (generally 0 to 60%, v/v). Solvent A was 0.05% TFA in triply distilled water and solvent B was acetonitrile adjusted with about 0.03% TFA to obtain a flat baseline at 214 nm during gradient elution. Both solvents were continuously degassed with helium.

Columns

The conventional column (75 × 4.6 mm), packed with TMS-250, was a gift from Dr. Y. Kato (Toya Soda, Tonda, Japan). This C_1 reversed-phase material has a particle size of 10 μm, with pores of 25 nm.

Microbore columns (50 × 1.0 mm) were made of stainless-steel tubing (1/16 in. O.D.). The ends of the column were sealed with 2-μm frits (1/16 in. × 0.6 mm, Alltech), which fitted in 1/16-in. Valco unions (bore 0.25 mm). The microbore columns were packed in the laboratory as follows: 50 mg of packing material in 2 ml methanol were sonicated for 5 min and then transferred into an empty column (300 × 4.6 mm) which was used as the slurry reservoir. After filling the reservoir with methanol, the microbore column was mounted on top of it. The column was then packed upwards by pumping methanol for 20 min at 1.5 ml/min.

Determination of peak volume and peak capacity

Taking the peak volume as four times the bandwidth[4,5] and the latter as equal to 0.425 times the peak width at half-height (assuming Gaussian shape), the peak volume was calculated as 1.7 times the peak width at half-height. Peak widths were measured in volume units at a suitable chart speed of the recorder. To obtain the peak capacity, the flow-rate was multiplied by the time the gradient would have taken if completed to 100% acetonitrile, t_G^0, and then divided by the peak volume[5].

RESULTS AND DISCUSSION

In this study we compared two columns of different dimensions (75 × 4.6 and 50 × 1.0 mm, respectively) packed with TMS-250, a wide-pore C_1 reversed-phase packing. Microbore columns packed with other wide-pore packings showed comparable or lower separation efficiencies for the proteins used in this study. The former packing was preferred, because also larger, more hydrophobic proteins can be eluted from this material with the low-viscosity acetonitrile–TFA solvent system[8].

In order to compare sensitivity and efficiency, both columns were loaded with a mixture of four proteins (ribonuclease, cytochrome *c*, carbonic anhydrase and ovalbumin) and subsequently eluted with a linear gradient of acetonitrile. Because of the large capacity factors of proteins, band broadening at the injection side is insignificant, since the proteins are initially concentrated on top of the column[5,6]. Therefore, to study the performance of the microbore column, a conventional injection valve was employed. However, to reduce analysis time, the volume between the switching valve of the low-pressure gradient system and column inlet was kept minimal (about 0.4 ml).

As for the applied detector, besides the standard flow cell (14 μl), a microflow cell (1.9 μl) was available, and the effect of cell volume on peak height was studied. It was found that relatively higher peaks were observed with the microcell only at flow-rates of 0.05 ml/min or less, that is with peak volumes below 40 μl. Therefore at first sight such flow-rates in combination with the microcell might be favourable. When however smaller amounts of protein (less than 1 μg) were eluted under these conditions it appeared that the detection sensitivity was limited by an increase in short-term noise, but more important by baseline distortions due to solvent-lens effects in the cell. This solvent-lens effect is caused by solvent gradients in the cell, which through differences in refractive index result in either convergency or divergency of the light beam directed to the photomultiplier[9]. It appeared that the design used for the standard flow cell, which strongly suppresses solvent-lens effects, could not be fully implemented for the construction of the microcell, due to the much smaller dimensions[10]. Therefore the application of this microcell seems to be indicated for situations where relatively large amounts of protein (micrograms) need to be separated with flow-rates below 0.1 ml/min. The remainder of this study was performed with the standard flow cell.

To study the effect of column diameter on peak height, standard proteins were eluted from both columns with gradients of 5% acetonitrile per min at several flow-rates. We are aware of the fact that column length will also contribute to the observed differences, but this effect will be relatively small compared with diameter contributions, which are proportional to the square of the diameter[5]. When express-

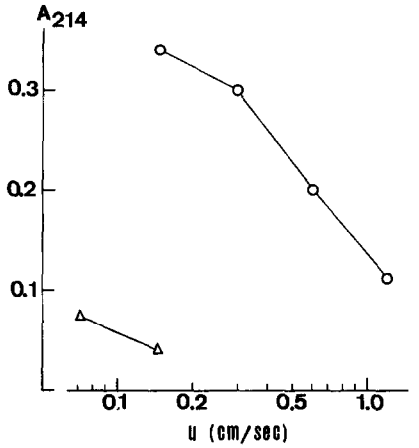

Fig. 1. Plot of peak height (absorbance units) *vs.* linear solvent velocity in the column *u*, for the conventional column (△, 75 × 4.6 mm) and for the microbore column (○, 50 × 1.0 mm), both packed with TMS-250. The linear velocity was calculated from the flow-rates (0.5, 1.0 and 0.05, 0.1, 0.2, 0.4 ml/min respectively) assuming a porosity factor of 0.7. The peak height was obtained as the mean of the individual values obtained for the four protein standards (see Fig. 5, 500 ng each) which were eluted with linear gradients of 5% acetonitrile per min.

ing the flow-rate as the linear velocity of the mobile phase moving through the column, it appeared that at the same linear velocity the peak heights of all four proteins were 8–9 times higher on the microbore column (Fig. 1). However, at this linear velocity the analysis time on the microbore column is much longer. Therefore, for practical reasons the peak height may be compared for that particular flow-rate at which the microbore column has about the same analysis time as the conventional column at its commonly used flow-rate of 1.0 ml/min. This is the case at 0.2 ml/min. Under these conditions the sensitivity of the microbore column is 5–6 times better.

Further support for the use of these flow-rates to compare peak heights can be obtained from the data shown in Fig. 2. As peak heights tend to increase, and resolution tends to decrease, at lower flow-rates while maintaining the gradient time[5], it was verified that the higher sensitivity on the microbore column was not obtained at the cost of resolution. Proteins were eluted from both columns at these flow-rates (0.2 and 1.0 ml/min, respectively) with different gradient times. The resolution was quantitatively measured by use of the peak capacity, that is the number of peaks with a resolution of unity that can fit in a chromatogram when the gradient is completed, that is carried out to 100% organic solvent concentration[5]. From Fig. 2 it is seen that the increased peak heights are a consequence of the microbore dimensions. Both columns when eluted at flow-rates of 1.0 and 0.2 ml/min respectively have comparable peak capacities or resolution over a range of gradient times. In addition, the peak volume ratio over this range remains almost constant (Fig. 2b). Therefore, when both columns were compared at flow-rates which resulted in equal resolution, the peak heights obtained with the microbore column were five times higher and the peak volumes, ranging between 40 and 110 μl, depending on protein and gradient time, were about five times smaller.

At this point it was relevant to know whether with the relatively short gradient

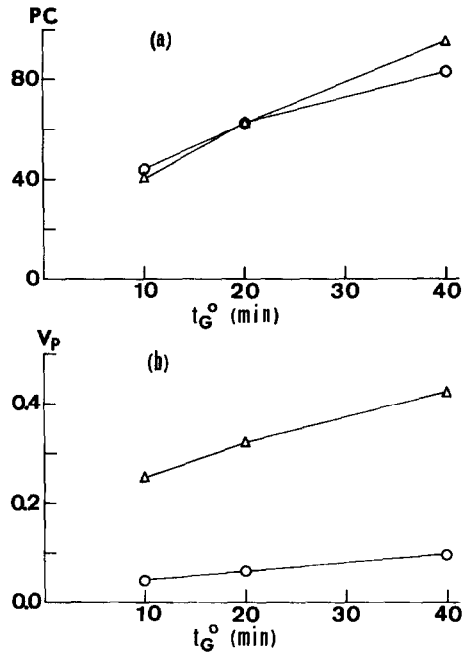

Fig. 2. Variation of peak capacity (PC) (a) and peak volume (V_p in ml) (b) with gradient time (calculated for completion to 100% acetonitrile), t_G^o. Both variables were based on the mean peak width at half-height observed for the four protein standards (see Fig. 5, 500 ng each) eluted from the microbore column (○) at 0.2 ml/min and from the conventional column (△) at 1.0 ml/min.

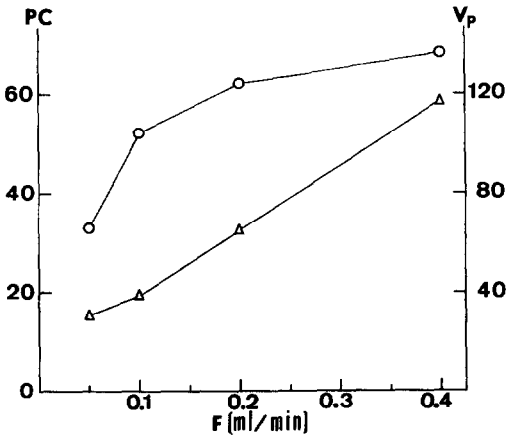

Fig. 3. Variation of peak capacity (PC, ○) and peak volume (V_p in μl, △) with the flow-rate, F, on the microbore column. Both variables were calculated from the mean peak width at half-height observed for the four protein standards (see Fig. 5, 500 ng each), eluted with linear gradients of 5% acetonitrile per min.

time (0–100% acetonitrile in 20 min) the abovementioned flow-rate of 0.2 ml/min was optimal with respect to resolution and sensitivity. From Fig. 3 it is seen that, because sensitivity and resolution are more or less inversely related, flow-rates between 0.1 and 0.2 ml/min will be optimal for both parameters. Above this range a large increase in peak volume is observed together with a small increase in resolution. Below 0.1 ml/min a strong decrease in resolution is associated with only a minor reduction of peak volume. Besides this, the peak asymmetry tended to increase at lower flow-rates (1.0–1.3 at 0.4 ml/min to 2.1–2.6 at 0.05 ml/min), which further reduced the effective resolution. The applied gradient has a steepness of 5% per min, which was predicted to be optimal for the size of this microbore column at a flow-rate of 0.2 ml/min[2,5].

Thus, the aforementioned conditions, that is a flow-rate of 0.1–0.2 ml/min and a gradient of 0 to 100% acetonitrile in 20 min, appear to be optimal for analytical use of the microbore column. Increasing either the gradient time or the flow will decrease sensitivity by an increase in peak volume, while the resolution only slightly increases (see Figs. 2 and 3). On the other hand a decrease in one of these parameters strongly decreases resolution with a minor improvement in sensitivity. A longer gradient time together with a lower flow-rate might be advantageous, but would drastically increase the total analysis time. However, this aspect is less important in situations concerning only one specific protein, where gradients covering a small difference in solvent strength can be used.

During the course of this study it was found that the sensitivity of detection was not only improved by smaller peak volumes, but also by a reduction in baseline noise. At more sensitive detector settings the baseline usually becomes disturbed by solvent peaks. A lower flow-rate reduces the amount of solvent contaminants absorbed by the column, resulting in a more stable baseline. This effect significantly contributed to an overall increase in sensitivity, which allowed us to detect proteins down to 1 ng (Fig. 4). This is about 20 times better than was previously possible with the conventional column.

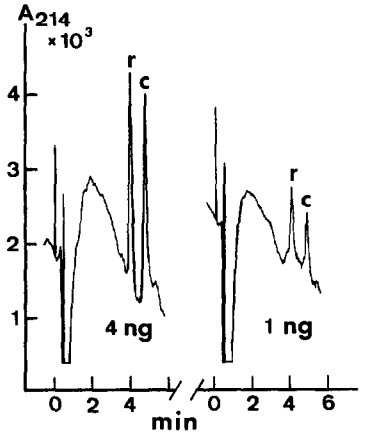

Fig. 4. Chromatography of 4 and 1 ng of ribonuclease (r) and cytochrome *c* (c) on the microbore column (50 × 1.0 mm), packed with TMS-250. Proteins were eluted with a linear gradient of 20–75% acetonitrile in 8 min at a flow-rate of 0.2 ml/min.

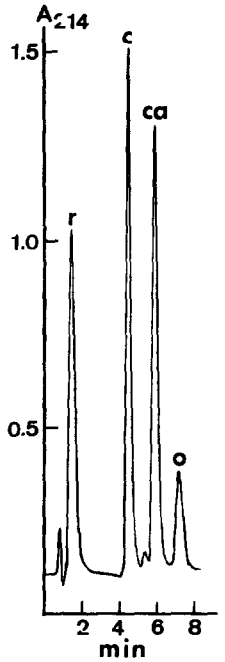

Fig. 5. Stepwise elution at 0.1 ml/min of ribonuclease (r), cytochrome *c* (c), carbonic anhydrase (ca) and ovalbumin (o) from the microbore column. After injection of the proteins (2.5 μg each) the solvent was directly changed from 30 to 60% acetonitrile.

Protein loss due to adsorption on the walls of connecting tubing as described by Trumbore *et al.*[11] was not observed during this study. The detector response was linear over the range studied (1–500 ng of protein). In another study[12] using the same equipment but with a conventional column (40 × 4.6 mm) we obtained a linear response from 40 ng to 5 μg. Probably the chemical nature of the mobile phase used in the present study, which may prevent aggregation and wall-coating behaviour[11], contributes to this. On the other hand, eventual losses due to handling after elution will be reduced by the small elution volume when compared with the use of conventional columns.

As it appeared that short steep gradients could be used for the elution of proteins from the microbore column at high resolution, we also investigated single step elution. Fig. 5 shows the chromatogram obtained when, after injection of the protein mixture, the solvent was changed in one step from 30 to 60% acetonitrile. The same pattern was found when the solvent change was delayed for a few minutes after injection, except for ribonuclease which was eluted isocratically. In this way very reproducible peak heights and retention times were obtained, but at a lower resolution than with gradient elution. No special active mixing device was used. Obviously a small, steep gradient is created by passive mixing in the lines between the switching valve and column inlet, and in the dual-piston pump, together having a volume of about 0.4 ml. Although this method of single-step elution leaves very little room for influencing the separation, it certainly is applicable to specific situations, especially when no gradient system is available.

Finally, peak capacity, described by Snyder and co-workers[5] for gradient elution, as applied in this study, proved to be an adequate tool for the quantitative comparison of separation efficiencies. This parameter can be obtained directly from the chromatogram, in contrast with the laborious procedure to obtain the plate number. The latter has to be derived from gradient elution data[5], since plate numbers based on isocratic data do not correlate with separation efficiency under gradient conditions[13].

CONCLUSIONS

Microbore reversed-phase HPLC was used effectively for the analysis of proteins down to 1 ng at moderate flow-rates. Except for the column, no special equipment was necessary for a considerable improvement in the sensitivity of detection. When compared with a conventional column, the peak volumes (40–110 μl) were five times smaller with the microbore column at equal chromatographic efficiencies and analysis time. The sensitivity was further amplified by a reduction in solvent peaks, which resulted in a 20-fold overall increase.

With respect to efficiency, sensitivity and analysis time, a flow-rate of 0.1–0.2 ml/min and gradients of 5% acetonitrile per min were found to be optimal.

Because of the small peak volume which can be obtained, the method might also be very useful to recover proteins from diluted solutions prior to subsequent analysis[6].

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