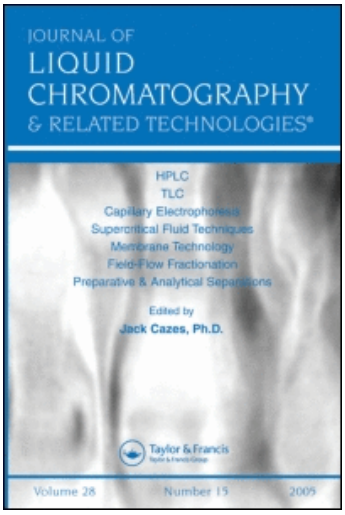

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Effects of Flow Rate and Eluant Composition on the High Performance Liquid Chromatography of Proteins

Barry N. Jones[a]; Randolph V. Lewis[a]; Svante Pääbo[a]; Kohichi Kojima[a]; Sadao Kimura[a]; Stanley Stein[a]
[a] Roche Institute of Molecular Biology, Nutley, New Jersey

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EFFECTS OF FLOW RATE AND ELUANT COMPOSITION ON THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

Barry N. Jones, Randolph V. Lewis, Svante Pääbo, Kohichi Kojima, Sadao Kimura and Stanley Stein

Roche Institute of Molecular Biology
Nutley, New Jersey 07110

ABSTRACT

The influence of eluant flow rate on efficiency in reverse-phase and ion-exchange high performance liquid chromatography of proteins was studied. Both isocratic and gradient elution led to the same conclusions. The use of flow rates much lower than those generally employed was found to be important for the efficient separation of large and small proteins. Several eluant systems were also examined to determine the effects these had on the efficiency of the reverse-phase separation of proteins.

INTRODUCTION

Over the last few years several techniques have been developed for isolating large peptides and proteins by high performance liquid chromatography (HPLC) in the gel permeation (1-4), affinity (5), ion-exchange (2,6,7), normal phase (8) and reverse phase (9-13) modes. For the best utilization of these techniques, the proper combination of chromatography conditions must be chosen. This selection includes such variables as the support, the eluting solvents, and the flow rate. Studies using gel permeation for protein chromatography have shown that the flow rate and eluting solvents have a large affect on column efficiency (4,7). This report extends these parameters to reverse-phase chromatography by documenting the importance of low flow rate and proper eluting solvents in achieving

the highest efficiency from columns used for protein separations. For this study a reverse phase C_8-column was chosen since it has been shown to be particularly useful for the HPLC of proteins (8,14). In addition, the influence of flow rate on column efficiency using the ion-exchange mode for protein chromatography was examined.

MATERIALS AND METHODS

Pyridine, acetic acid, formic acid, and n-propanol used for column elution were distilled over ninhydrin. Phosphoric acid was used without further purification. High purity water was obtained with a system from Hydro Service and Supplies (Durham,NC). Phenylalanine, aspartic acid, cytochrome C and ribonuclease were from Sigma (St. Louis, MO). Bovine serum albumin was obtained from Miles Laboratories, Inc. (Elkhart, IN) and chymotrypsinogen from Pharmacia Fine Chemicals (Piscataway, NJ). Collagen α_1 chain (rat) was a gift of Dr. Anthony Fallon.

The chromatography system consisted of a high-pressure Milton Roy minipump (Lab Data Control, Riviera Beach, FL) and a sample injection valve with a 20 µl or 1 ml loop (Rheodyne, Berkeley, CA). The following 25 x 0.46 cm columns were used: Lichrosorb RP-8 (10 µm particle size, 10 nm pore diameter, Ace Scientific, Edison, NJ), Ultrasphere-Octyl (5 µm particle size, 10 nm pore diameter, Altex Scientific, Berkeley, CA), Lichrosphere C_8 (10 µm particle size, 50 nm pore diameter) (15), and Carboxymethyl (CM)-Glycophase (10 µm particle size, 10 nm pore diameter) (16). Gradients were generated with either an Ultrograd (LKB, Hicksville, NY) or a Chrontrol unit (17).

The column effluent was monitored automatically for amino acids, peptides and proteins with fluorescamine (Hoffmann-La Roche, Nutley, NJ) using a stream-sampling technique as previously described (18). The following considerations are related to the suitability of this system for calculating column efficiency at different flow rates. The column outlet was connected to the stream-sampling valve with a short (1 cm) length of 0.3 mm (inner diameter) tubing. With this

type of stream sampling, there was no contribution from the column to the total flow rate of the monitoring system. The delay time (40 sec) for the fluorogenic reaction was taken into account for determining the elution time from the column. The high flow rate (60 ml/hr) of the monitoring system, in conjunction with the small flow cell volume (45 µl), resulted in minimal peak broadening. Further experimental details are given in the text and in the figure legends.

RESULTS AND DISCUSSION

The efficiency or gross resolving power of a chromatographic column can be expressed in terms of theoretical plates (N) which is related to the ratio of the retention time of a solute under isocratic conditions to its peak width. Thus, the greater the plate count, the better the column will perform. The height equivalent to a theoretical plate (HETP) can also be used to express column efficiency and is obtained by dividing the length of the column by the theoretical plate count. In the present work, theoretical plate counts were determined by injecting the sample onto the column in a small volume (20 µl) and then eluting isocratically. The resulting plate counts were then converted to HETP values. Plate counts from gradient elution were also calculated using the above methodology. These numbers are clearly not true plate counts, but can be useful for comparing different gradients and solvents.

Figure 1 depicts the general phenomenon of decreased column efficiency with increasing eluant flow rate. At all flow rates examined on this 10 nm pore C_8 reverse-phase support, the large protein, bovine serum albumin (BSA: 68,000 daltons), did not chromatograph as efficiently as the small protein, ribonuclease (13,700 daltons), and neither protein chromatographed as efficiently as the amino acids, aspartic acid (133 daltons) and phenylalanine (165 daltons). This decreased efficiency for proteins is due in part to the fact that large molecules have much lower diffusion rates than do low molecular compounds and equilibrate much more slowly

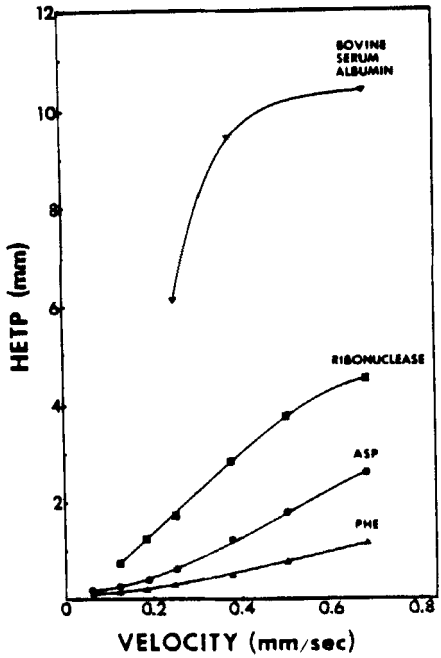

Figure 1. Effect of mobile phase velocity on column efficiency under isocratic conditions. Aspartic acid and phenylalanine were eluted from a 10 nm pore C_8-column (10 μm particle size) with 1M pyridine-0.5M acetic acid (pH 5.5). Ribonuclease was eluted with the same buffer but containing 2% n-propanol while bovine serum albumin was eluted with this buffer containing 20% n-propanol. Each point represents the average of three determinations. With this system a linear velocity of 0.5 mm/sec corresponds to a flow rate of approximately 20 ml/hr.

with the stationary phase. Consequently, the plate height of the column appears to approach a limiting value for all substances as the flow rate is decreased.

The very poor column efficiency obtained on chromatography of BSA (Figure 1) is also the result of its poor penetration into the 10 nm pores of the column support. However, by using a 50 nm pore C_8-support (10 μm particle size), increased efficiency can be obtained for large proteins (15). As seen in Figure 2, proteins from 12,500 to 95,000 MW behave very similarly

on the 50 nm pore reverse-phase support and as in Figure 1, a limiting value is approached at the lower flow rates.

The effect of flow rate on column efficiency was also examined for a 10 nm pore C_8-support with a 5 μm particle size. Peptides and small proteins were found to chromatograph very efficiently on this column. This increased efficiency is due mainly to the small particle size of the support with some contribution from the higher carbon content due to monomer coating of this particular column. The results obtained for the chromatography of cytochrome C at various flow rates with this support are shown in Figure 2.

Studies with ion-exchange HPLC with proteins have indicated that flow rate can affect column efficiency (2). However, the flow rates used in these studies (3 ml/min) were higher than those found to be optimal for the reverse-phase columns. Therefore, the effect of flow rate on the efficiency of an ion-change support was investigated. Using a CM-glycophase support, a large increase in column efficiency was obtained for various proteins by lowering the flow rate. This is illustrated in Figure 2 which shows the results obtained for the chromatography of cytochrome C on this column.

For protein and peptide purification, samples are usually injected into the column in relatively large volumes (1 ml) in a weak eluant and the HPLC column is actually used to concentrate the sample. Furthermore, gradient elution is then used so that substances with widely differing affinities for support can be resolved in a single elution. The influence of eluant flow rate on column efficiency under these conditions was investigated for the reverse-phase mode. A mixture of ribonuclease and chymotrypsinogen was chromatographed at a flow rate of 15 ml/hr using a 60 min linear gradient. This was repeated with the combinations of 30 ml/hr with a 30 min gradient and 60 ml/hr with a 15 min gradient. In this way, the flow rate was varied while the gradient was essentially unchanged. The

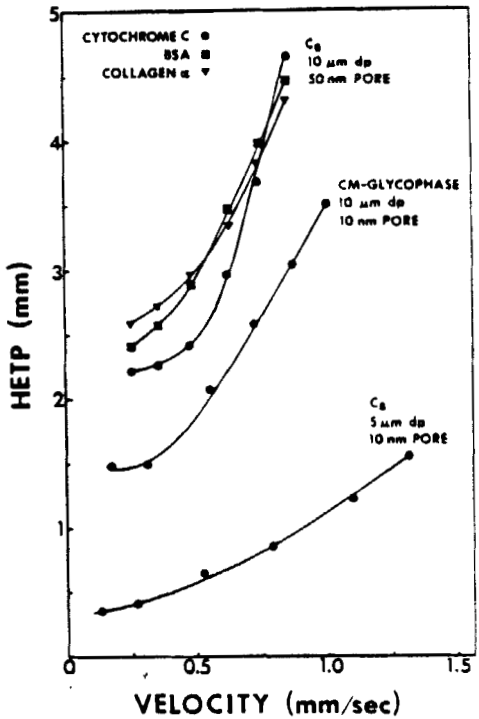

Figure 2. Effect of mobile phase velocity on column efficiency under isocratic conditions. Proteins were eluted from two reverse-phase C_8-columns that differed in both particle size (dp) and pore diameter. The buffer system was 0.5M formic acid-0.4M pyridine (pH 4.0) using n-propanol concentrations of 20% (collagen α_1), 24% (cytochrome C), and 26% (BSA). Cytochrome C was also eluted isocratically at various flow rates from a carboxymethyl (CM)-Glycophase ion-exchange column using 0.36M acetic acid-0.09M pyridine (pH 4.0). For the above systems, a flow rate of 20 ml/hr resulted in a linear velocity of approximately 0.5 mm/sec.

resulting chromatograms are presented in Figure 3 and indicate that the proteins eluted at the same positions under all three conditions. However, they eluted in smaller volumes as the flow rate decreased and were thereby best resolved with the lowest flow rate. In fact, reducing the flow rate four-fold (from 60 to 15 ml/hr) resulted in approximately a 50% reduction in the peak width at the half height.

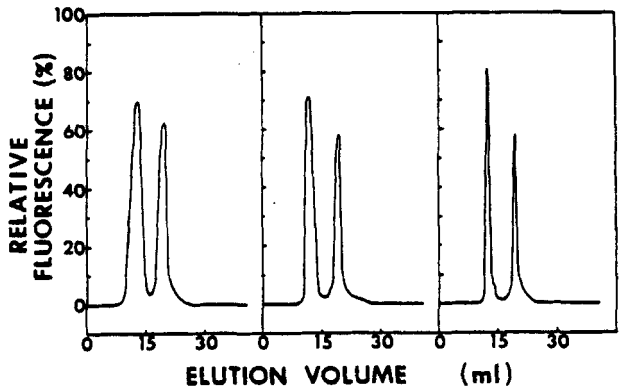

Figure 3. Effect of eluant flow rate on gradient elution. A mixture of ribonuclease and chymotrypsinogen was chromatographed on a 10 nm, 10 μm dp C_8-column under the following conditions: Flow rates and run times were 60 ml/hr - 15 min, 30 ml/hr - 30 min, and 15 ml/hr - 60 min for the left, center and right panels, respectively. The column was eluted with 0.5M formic acid-0.14M pyridine (pH 3.0) using a linear gradient from 0 to 40% n-propanol. Ribonuclease elutes before chymotrypsinogen.

To further investigate column efficiency, three different solvent systems, 0.1% phosphoric acid, 0.1% formic acid, and 0.5M formic acid-0.4M pyridine, were compared using n-propanol gradient elution. There were differences in the elution times of various proteins with the different solvent systems although they were generally less than 10%. To further characterize these solvent systems the number of theoretical plates/column was calculated at various flow rates using cytochrome C. The gradient and flow rate were varied as in Figure 3 to give equal volume gradients at all flow rates. The resulting plate numbers are derived using gradient not isocratic elution and therefore are not true plate counts, but serve an illustrative purpose. The three systems show large differences in the plate count (Figure 4), which are accentuated as the flow rate is decreased. The phosphoric acid system produces the lowest plate count values and these values are affected very little by differences in flow rate. Formic acid gives higher plate count counts than phos-

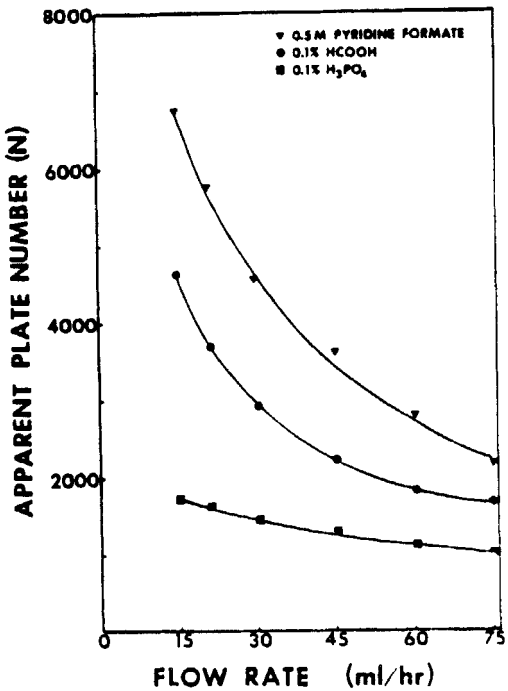

Figure 4. Effects of flow rate and solvent system on column efficiency. Cytochrome C was eluted on a 50 nm pore C_8-column (10 µm dp) using a linear n-propanol gradient at the flow rates indicated. The gradient was adjusted to the flow rate in order to provide equal volume gradients in all cases. 0.5M formic acid-0.4M pyridine (pH 4.0), 0.1% formic acidm and 0.1% phosphoric acid were the solvent systems used. Apparent plate counts were obtained using the peak width at the half height. All points are the average of three runs.

phoric acid at all flow rates and shows a clear decrease as the flow rate increases. Although not shown, acetic acid gave results similar to formic acid. The formic acid-pyridine buffer yielded the highest plate counts but also showed the greatest effects of increasing flow rates. With BSA as the eluting protein, the same qualitative results were obtained with these three solvent systems although lower plate counts were found at these low pH values probably due to the aggregation of BSA (19).

It is clear that increased resolution can be attained by decreasing the eluant flow rate in HPLC. What is sacrificed is the time required for the chromatographic run. In previous reports using the reverse phase and ion-exchange modes of HPLC with proteins, flow rates of 60 to 180 ml/hr were generally employed (2,11,13,20). In contrast, we have found that a gradient of 2 hr with a flow rate of 20 ml/hr to be a good compromise for protein and peptide chromatography. Other combinations have also been used effectively depending on the resolution needed.

The data obtained for the various solvent systems clearly can be applied only to reverse-phase supports. They indicate that in the reverse-phase chromatography of proteins the solvent system used can greatly affect column efficiency. It seems advisable to test various solvent systems when setting up to chromatograph a new protein, with the formic acid-pyridine system as a good first choice. Other positive counter ions such as N-methyl morpholine can be used if UV detection is employed.

In summary, it is possible to obtain excellent resolution of proteins by reverse-phase and ion-exchange HPLC. To obtain the maximum resolution, flow rates considerably lower than those generally used in HPLC are necessary. The solvent system can also greatly affect resolution in the reverse-phase mode and should be varied to obtain maximum resolution. With these factors optimized, it has been demonstrated in several instances (10,14,15,21) that previously difficult or impossible protein purifications can be achieved readily with HPLC.

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