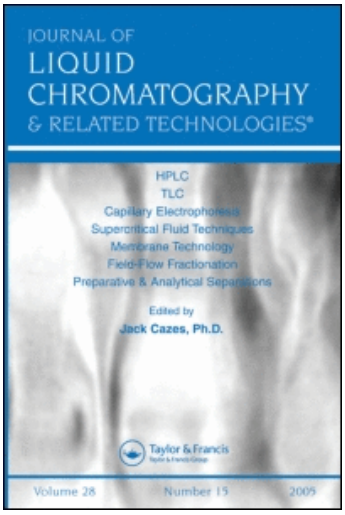

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Factors Influencing Fast Liquid Chromatography of Proteins and Peptides with Microbore Technology

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To cite this Article Kawakatsu, Miyako and Gooding, Karen M.(1993) 'Factors Influencing Fast Liquid Chromatography of Proteins and Peptides with Microbore Technology', Journal of Liquid Chromatography & Related Technologies, 16: 1, 21 — 32

To link to this Article: DOI: 10.1080/10826079308020894

URL: http://dx.doi.org/10.1080/10826079308020894

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(1), 21-32 (1993)

FACTORS INFLUENCING FAST LIQUID CHROMATOGRAPHY OF PROTEINS AND PEPTIDES WITH MICROBORE TECHNOLOGY

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ABSTRACT

Microbore columns (1 and 2.1 mm I.D.) were investigated for their utility in fast liquid chromatography of proteins and peptides. Using a series of SynChropak large pore reversed phase columns, the effects of flowrate and gradient on resolution of standard protein and peptide mixtures were determined. Excellent resolution could be achieved on 2.1 mm I.D. columns with analysis times under five minutes.

INTRODUCTION

High performance liquid chromatography has become a method of choice for the analysis of proteins and peptides in milligram quantities or less due to its superior resolution, selectivity, sensitivity and speed. One refinement of the technique is to use systems which can achieve the separations in a few minutes rather than the thirty to sixty minutes commonly employed. The principle methods for achieving these fast analyses have been short columns

of 1-3 μm nonporous supports (1,2) and perfusive supports (3). Large pore (4000Å) supports in 1 mm I.D. columns on a specialized system have also been used (4).

This paper will show that small bore columns of 5-10 μm HPLC supports can also yield very fast analyses with excellent resolution if they are run under optimized conditions. In addition to the speed, microbore columns (1 - 2.1 mm I.D.) have advantages over standard 4.6 mm I.D. columns in terms of solvent consumption and sensitivity.

MATERIALS

Chemicals

Standard proteins, ribonuclease A, cytochrome c, lysozyme, transferrin and bovine serum albumin, were purchased from Sigma Chemical Company (St. Louis, MO). The peptide standard for reversed phase was from SynChrom, Inc. (Lafayette, IN). Acetonitrile, HPLC-grade water and trifluoroacetic acid (sequenal grade) were purchased from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan).

Apparatus

Gilson Model 305 HPLC pumps with 5SC heads, Model 805 manometric module and Model 811B mixer were used. The mixer was equipped with a 1.5 ml chamber except for studies with 1 mm I.D. columns which employed a 65 μl chamber. A Gilson Model 116 detector was used at 210 nm. The detector had a 5 mm light path and 12 μl cell except for studies with 1 mm I.D. columns which used a cell with a 2 mm light path and a 1.6 μl volume. The gradient system was controlled and the detector signals were collected and processed using a Gilson Model 715 system

controller. A Rheodyne Model 7125 injector was used with a 100 µl sample loop (0.25 mm I.D. stainless steel) made in-house. An Upchurch (Oak Harbor, WA) precolumn filter (A-318) was attached before the analytical columns.

SynChropak RP-8, RP8-1000, RPP-1000 and RPP-4000 columns were obtained from SynChrom, Inc. (Lafayette, IN).

METHODS

Standard protein mixtures (1. ribonuclease A, 2. cytochrome c, 3. lysozyme, 4. transferrin, 5. bovine serum albumin) were prepared to be 400 ng/µl for each protein except cytochrome c, which was 200 ng/µl at its final concentration. Unless specified, 6 µl of the protein solution was injected. The peptide solution was reconstituted in water to be 100 ng/µl.

Gradients were run from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 59% acetonitrile.

To properly compare resolution at widely varying flowrates on a 2 mm I.D. column, the extra volume of the HPLC system was evaluated. Tubing lines from the column inlet to the column outlet were directly connected In the above system, including the 100 µl sample loop without a column. Using 0.5% acetone and water as mobile phases, the extra volume, or the point that the linear gradient started, was determined to be 3.5 ml.

RESULTS AND DISCUSSION

Column Diameter

The relative performance of 1 mm and 2.1 mm I.D. microbore columns for protein mixtures was evaluated at similar linear velocities on SynChropak RPP-4000, a 10 µm reversed phase silica

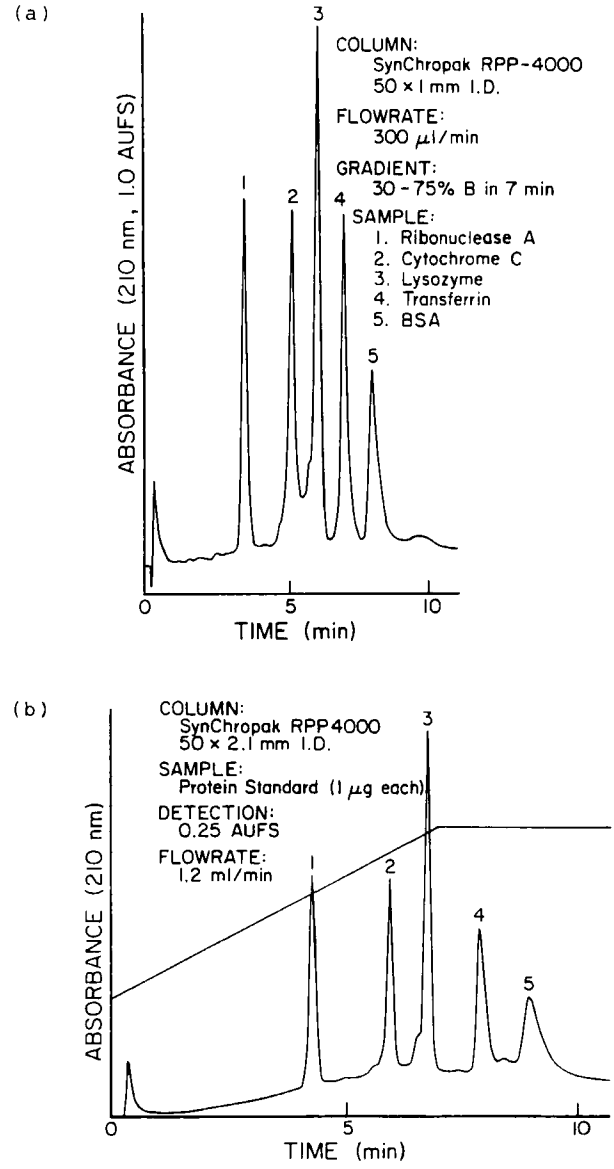

FIGURE 1. The effect of column diameter on resolution of proteins. Column: SynChropak RPP-4000. Gradient: 30-75%B in 7 min. Sample: Protein standards. (a) 50 x 1mm I.D. Flowrate: 300 µl/min. (b) 50 x 2.1mm I.D. Flowrate: 1.2ml/min.

EFFECT OF FLOWRATE ON PEAK WIDTH AND SENSITIVITY

Peak Name	Flow Rate (mL/min)	RT (min)	Width (1/2) (sec)	Height (mV)	% Lyz
RNASE A	0.25	24.9	15.5	161.1	55
	0.5	20.5	12.6	93.3	46
	1.0	18.6	14.0	46.9	42
	1.5	17.5	9.8	49.3	64
	3.0	16.4	11.2	20.2	52
CYT C	0.25	28.6	19.0	114.4	39
	0.5	23.9	14.1	93.8	47
	1.0	21.7	11.9	47.7	43
	1.5	20.9	11.2	32.9	43
	3.0	20.0	11.2	16.4	42
LYZ	0.25	30.2	18.3	293.8	100
	0.5	25.7	14.8	201.7	100
	1.0	23.5	12.6	111.1	100
	1.5	22.6	13.3	77.0	100
	3.0	21.6	13.3	39.2	100
TRANS	0.25	32.1	22.5	140.1	48
	0.5	27.8	17.6	88.4	44
	1.0	25.8	14.1	52.2	47
	1.5	25.0	14.0	35.9	47
	3.0	24.3	15.5	19.0	48
BSA	0.25	33.8	61.8	96.7	33
	0.5	29.5	66.7	51.0	25
	1.0	27.6	33.0	30.9	28
	1.5	26.9	30.9	21.4	28
	3.0	26.3	28.1	11.2	29

SynChropak RPP-1000 (50mm x 2mm I.D.)

support with a C-18 ligand and 4000Å pore diameter. The 2.1 mm I.D. column was run with standard HPLC hardware as noted in METHODS. Because of the serious effects of extra-column volume on resolution for 1 mm I.D. columns, the system for the 1 mm column utilized a 65 µl mixing chamber and a 1.6 µl detector cell with a 2 mm light path. As is seen in Fig. 1, the resolution of the

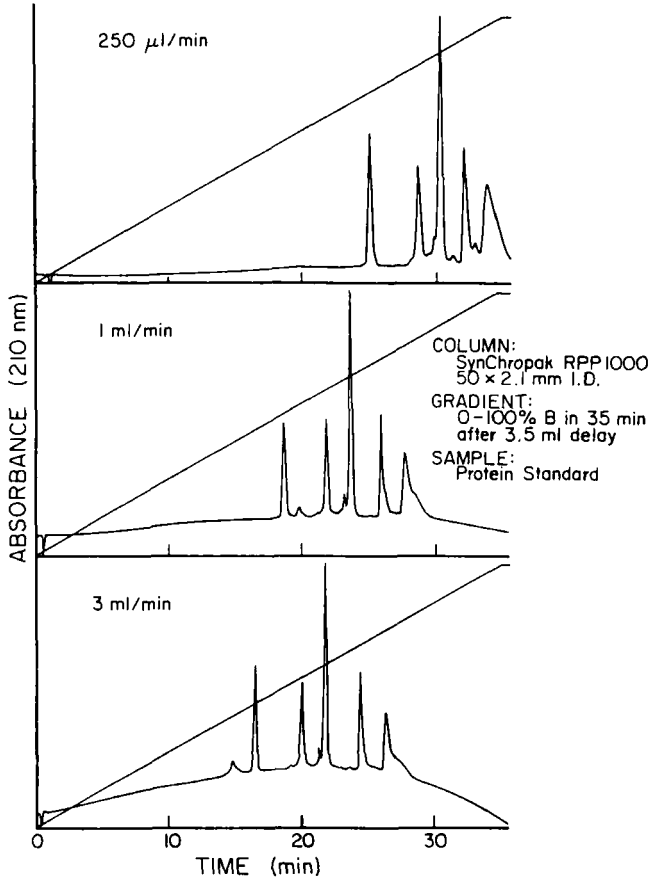

FIGURE 2. The effect of flowrate on resolution of proteins. Column: SynChropak RPP-1000, 50 x 2.1mm I.D. Gradient: 0-100%B in 35 min after 3.5ml hold. Sample: Protein standards.

proteins was better on the 2.1 mm column despite the use of a low volume system for the 1 mm column. The sensitivity was fourfold higher on the 1 mm I.D. column. Nonetheless, columns of 2.1 mm I.D. were chosen for the remainder of the study due to the ease of their operation and excellent resolution with standard HPLC hardware.

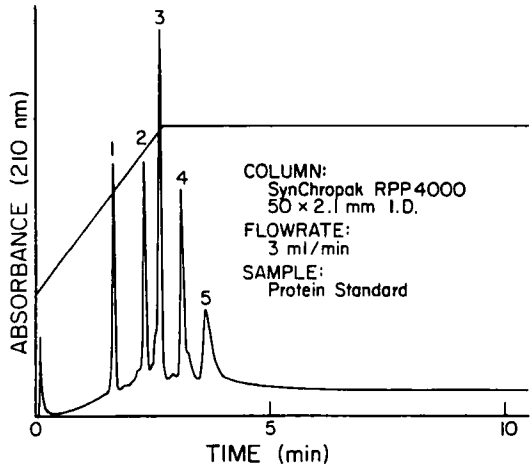

FIGURE 3. Effect of flowrate with constant gradient slope on resolution of proteins. Column: SynChropak RPP-4000, 50 x 2.1mm I.D. Flowrate: 3ml/min. Gradient: 30-75%B in 2.5 min. Sample: Protein standards.

Flowrate and Gradient

The effect of flowrate on resolution of proteins was examined on a 50 x 2.1 mm I.D. column of SynChropak RPP-1000, a 7 µm reversed phase silica support with a C-18 ligand and 1000Å pores. Flowrates ranging from 0.25 - 3.0 ml/min were used in this study. Because this range is twelvefold, the linear gradient was initiated after the 3.5 ml volume of the mixer and tubing. This delay ranged from 14 min at the lowest flowrate to 1.17 min at the highest. A 35-min linear gradient was initiated after the 3.5 ml hold. Table 1 shows the effect of flowrate on peak width for each of the standards while Fig. 2 illustrates representative chromatograms. It can be seen that the resolution is poorest at the lowest flowrate, 250 µl/min (linear velocity of 1.2 mm/s). Resolution improved with increased linear velocity and was still

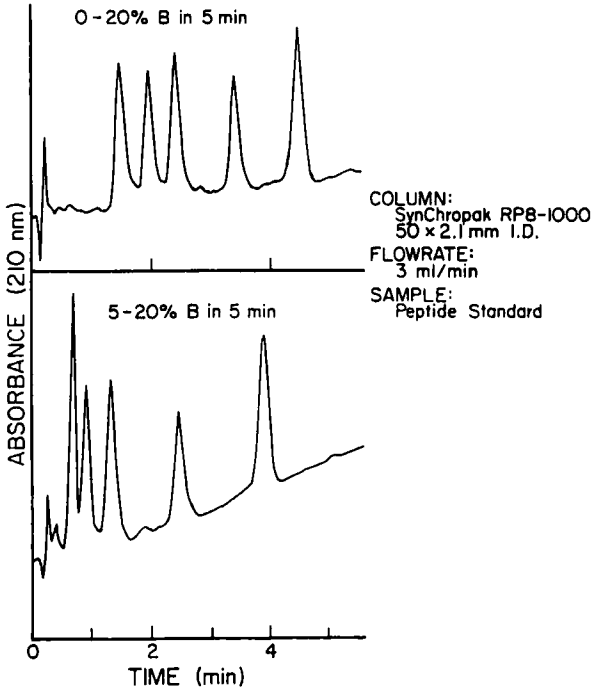

FIGURE 4. Effect of gradient on resolution of peptides. Column: SynChropak RP8-1000, 50 x 2.1mm I.D. Flowrate: 3ml/min. Sample: Peptide standard.

excellent at 3 ml/min (14.4 mm/s). Differences in relative area are at least partially explained by heterogeneity of some of the proteins, notably BSA.

Because the gradient time was kept constant in the previous experiments and the flowrate was varied, the gradient volume was different in each run. To insure that the effects of flowrate were also valid with constant gradient slope, the protein standard was rerun on the column seen in Fig. 1b with the flowrate increased 2.5-fold and the gradient volume kept constant at 8.4 ml. Fig. 3 shows that the resolution was maintained under

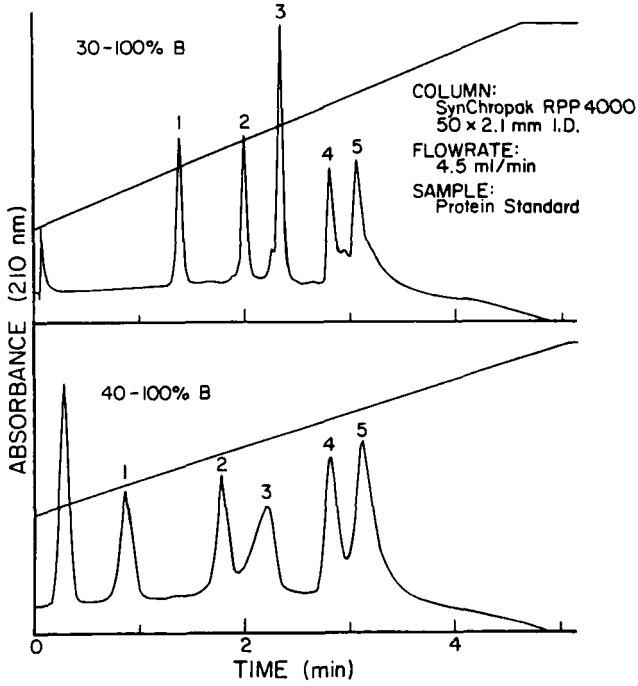

FIGURE 5. Effect of initial conditions on resolution of proteins. Column: SynChropak RPP-4000, 50 x 2.1mm I.D. Flowrate: 4.5ml/min. Sample: Protein standards.

these conditions which reduced the analysis time to less than four minutes. The total back pressure of this 2.1 mm I.D. column and system is under 2000 psi at 3 ml/min; therefore, these high flowrates are reasonable.

If optimum resolution is to be achieved in fast HPLC analyses, the gradient must be chosen carefully. The resolution of the five peptide standards seen in Fig. 4 is best when the initial conditions are weak enough to cause total binding to the support, as in Fig. 4a, rather than when the first components are partially eluted under the initial conditions, as in Fig. 4b.

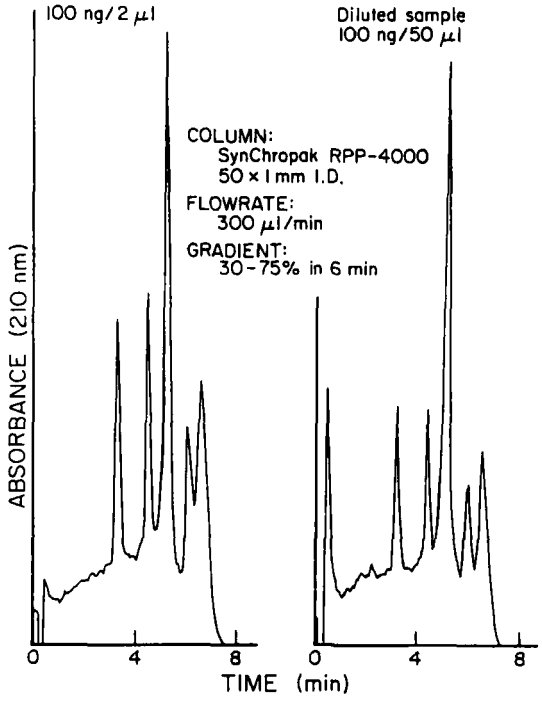

FIGURE 6. Effect of sample dilution on resolution. Column: SynChropak RPP-4000, 50 x 1mm I.D. Flowrate: 300 µl/min. Gradient: 30-75%B in 6 min. Sample: Protein standards.

This effect is more dramatic with proteins. As seen in Fig. 5, peak shape and resolution is best when initial solvent conditions bind all the proteins (Fig. 5a). There is little difference if the initial conditions are either 20 or 30%; however, beginning the gradient at 40% results in broad peaks and poor resolution (Fig. 5b).

Injection Volume

The use of gradient conditions for microbore columns minimizes deleterious effects caused by injection volume or

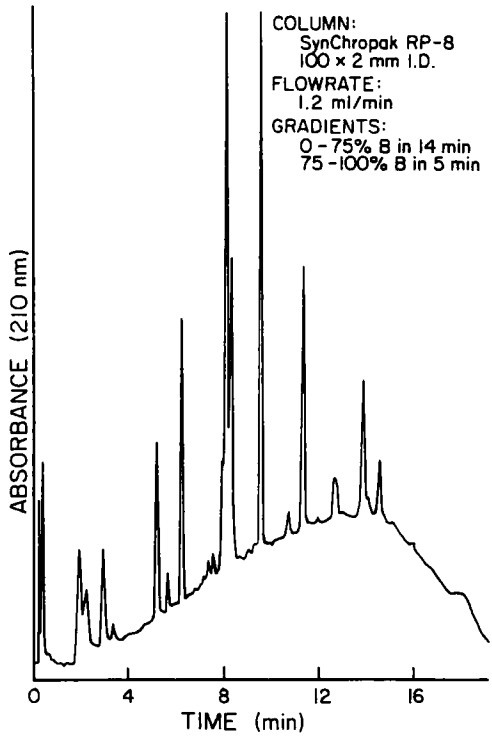

Figure 7. Fast analysis of tryptic digest. Column: SynChropak RP8, 100 x 2.1mm I.D. Flowrate: 1.2ml/min. Gradient: 0-75%B in 14min, 75-100%B in 5 min.

configuration. Fig. 6 illustrates that the resolution of protein standards on a 1 mm I.D. column was not diminished by a twenty-five fold increase in volume which was greater than the column volume. The lower peak heights for ribonuclease A and cytochrome c may be due to low level adsorption of these cationic proteins to the glass syringe.

Complex Mixtures

The applicability of this technique to more complex mixtures was evaluated with a tryptic digest of hemoglobin. A 10 cm column

was used to increase resolution for this analysis. The support was SynChropak RP-8, which has a C-8 ligand and 300Å pores. Mass transfer on this 300Å support did not appear to be a problem at this flowrate because excellent resolution was observed, as seen in Fig. 7. Further studies are ongoing to evaluate the role of pore diameter and ligand chain length in fast protein HPLC.

CONCLUSIONS

Fast protein liquid chromatography can be achieved without restrictive hardware constraints by using 50 x 2.1 mm I.D. columns packed with standard 5-10 µm reversed phase supports. Flowrates of 3 ml/min or linear velocities of 14.4 mm/s can yield separations in less than five minutes at pressures under 2500 psi without operational problems. Gradients should be formed with initial conditions which totally bind all of the components of the mixture.

ACKNOWLEDGEMENTS

The authors thank Hiroto Kotaniguchi for his help and very useful suggestions for this research.

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Received: May 1, 1992
Accepted: June 5, 1992