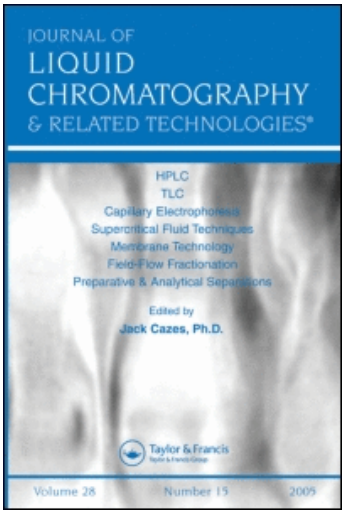

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THE EFFECTS OF PORE DIAMETER AND LIGAND CHAIN LENGTH ON FAST LIQUID CHROMATOGRAPHY OF PROTEINS AND PEPTIDES

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ABSTRACT

Fast flow separations of proteins and peptides were successfully carried out on microbore columns containing reversed phase supports of three pore diameters. Increasing flowrates from 0.25 to 3 ml/min (1.2 mm/s - 14.4 mm/s) did not show any adverse effects on the separations when SynChropak RPP-1000 and RPP-4000, which have 1000Å and 4000Å pore diameters, were used. At 3 ml/min, the 1000Å and 4000Å pore diameters yielded narrower peaks for proteins than the 300Å support, whereas the latter exhibited better resolution for peptides. Analyses in less than five minutes were achieved.

A series of ligand chains (C-4, C-8 and C-18) showed few differences in retention or resolution for either protein or peptide standards. Longterm stability of the 300Å and 4000Å supports was in excess of 25,000 column volumes when run at 3 ml/min with 0.1% trifluoroacetic acid.

INTRODUCTION

A common assumption in HPLC is that increasing the flowrate or linear velocity will concommitantly decrease resolution by increasing peak widths. This is based on the van Deemter/Knox equation: $h = Av^{1/3} + B/v + Cv$. For proteins and peptides, the C or mass transfer term is primarily responsible for band spreading because the diffusion coefficients are so small, making $v = ud_p/D_m$ large (1). In these equations, h is the reduced plate height, v is the reduced linear velocity, u is the linear velocity, d_p is the particle diameter and D_m is the diffusion coefficient of the solute in the mobile phase. In size exclusion chromatography, the adverse effects of high linear velocity on peak width increase with the molecular weight of the solute, as would be expected (2). To the contrary, in reversed phase chromatography, general improvement in peptide resolution with increased flowrate has been observed (3). Denaturing and renaturing of proteins during reversed phase chromatography has also been seen to be flowrate dependent (5).

High flowrates have been used successfully to achieve fast separations by high performance liquid chromatography. Fast analyses have been demonstrated with 1-5 µm nonporous supports (4, 6-8), perfusive supports (9, 10) and large pore supports (11, 12). Short columns were essential to many of these applications so that rapid analysis times could be achieved without excessive back pressures.

The use of microbore columns to cut solvent consumption and to increase sensitivity and recovery has seen increasing popularity, particularly for the analysis of submicrogram samples (13-17). It was recently demonstrated that proteins and peptides could be separated in five minutes or less on microbore reversed phase columns run at 3 ml/min

(12). That study suggested that supports with 1000Å or 4000Å average pore diameters may be optimal for the technique. This report describes a study of the effects of pore diameter on the resolution of protein and peptide mixtures by reversed phase chromatography in microbore columns at high linear velocities. The effects of ligand chain length on resolution under these conditions are also examined. Because the linear velocities are considerably higher than those normally used for 2.1 mm I.D. columns, the stability of the columns was also tested.

EXPERIMENTAL

Chemicals

Ribonuclease A (MW 13,700), cytochrome c (MW 12,500), lysozyme (MW 14,400), transferrin (MW 81,000) and bovine serum albumin (BSA) (MW 68,000) were purchased from Sigma Chemical Company (St. Louis, MO). The decapeptide standard was from Synthetic Peptides Incorporated (Edmonton, Canada). Acetonitrile was from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol and isopropanol were from Baxter Scientific Products (McGaw Park, IL). Trifluoroacetic acid (TFA) was from Pierce Chemical Co. (Rockford, IL) or WAKO Pure Chemical Industries, Ltd. HPLC-grade water was from Baxter Scientific Products or WAKO Pure Chemical Industries, Ltd.

Methods

Gilson Model 303 or 305 pumps were used. The mixer was equipped with a 1.5 ml chamber. The acetonitrile experiments employed a Gilson Model 116 detector with a 12 µl cell. The alcohol experiments used an ISCO Model V^4 detector with a 3.5 µl cell. The systems were

controlled and data collected with a Gilson Model 715 data system. Rheodyne Model 7125 injectors were used.

SynChropak silica-based reversed phase columns (50 x 2.1mm) were used (Lafayette, IN). SynChropak RP-4, RP-8 and RP-P columns contained 6.5 µm particles with 300Å pores and C-4, C-8 and C-18 ligands. SynChropak RP4-1000, RP8-1000 and RPP-1000 were 7 µm with 1000Å pores and the same ligands. SynChropak RP4-4000, RP8-4000 and RPP-4000 were 10 µm with 4000Å pores and the same ligands. The C-18 and C-8 bonded phases were monomeric and the C-4 was polymeric.

Methods were as described previously for the acetonitrile experiments (12). The 35 min linear gradient was programmed to begin after the 3.5 ml volume of the mixer and tubing had passed. The alcohol gradients went from 0.1% TFA in water to 0.05% TFA in methanol or isopropanol and were not adjusted for the dead volume. The methanol gradient went from 10% to 100% in five minutes and the isopropanol gradient went from 0 - 15% in five minutes.

RESULTS AND DISCUSSION

Effect of Pore Diameter on Protein Resolution

It was previously shown that excellent resolution of five standard proteins could be maintained on microbore columns of 2.1 mm I.D. at flowrates of 3 ml/min (12). The system employed a gradient delay of 3.5 ml and 35 min gradients at varied flowrates from 0.25 ml/min (1.2 mm/s) to 4.5 ml/min (21.6 mm/s). Resolution by reversed phase chromatography was compared for octadecylsilyl (C-18) supports with 300Å, 1000Å and 4000Å pore diameters (12). The delay time differed by 12- to 18-fold,

depending on the flowrate. On the 4000Å support, the resolution of the mixture was better at 3 ml/min than at 0.25 ml/min. The gradient conditions were the same for each pore diameter, although the design of the experiment resulted in a different gradient volume for each flowrate. The gradient time was kept constant for these experiments; therefore, the slope of the gradient in %B/min was constant but the volume or (gradient time x flowrate) increased with flowrate.

Figure 1 compares the resolution of proteins on supports with three different pore diameters under identical conditions at a flowrate of 3 ml/min (14 mm/s linear velocity). Under these conditions, resolution on the 300Å support was definitely inferior to that of the 1000Å and 4000Å packings despite the larger particle diameter of the latter. As seen in the graphs in Figures 2a and 2b, resolution was similar on all three columns at 0.25 ml/min, a standard flowrate for 2.1 mm I.D. columns. As the flowrate was increased, resolution remained constant on the 300Å support but increased on the larger pore materials. This is more dramatic for the protein pair of lysozyme and transferrin than lysozyme and cytochrome c. Transferrin has a molecular weight of 81,000 Daltons whereas the other two proteins are smaller than 20,000 D.

Figure 2c illustrates that the resolution of transferrin and BSA did not change significantly with flowrate on any pore. It was observed that the peak width for BSA increased with flowrate on the 300Å support whereas it decreased slightly on the 1000Å and 4000Å supports, as shown in Figure 3. Despite this fact, the resolution on the 4000Å support was slightly less because the retention of BSA was 3-6% lower than on the other two supports. BSA also exhibited some heterogeneity under these conditions, as seen in Figure 1, giving higher and more variable

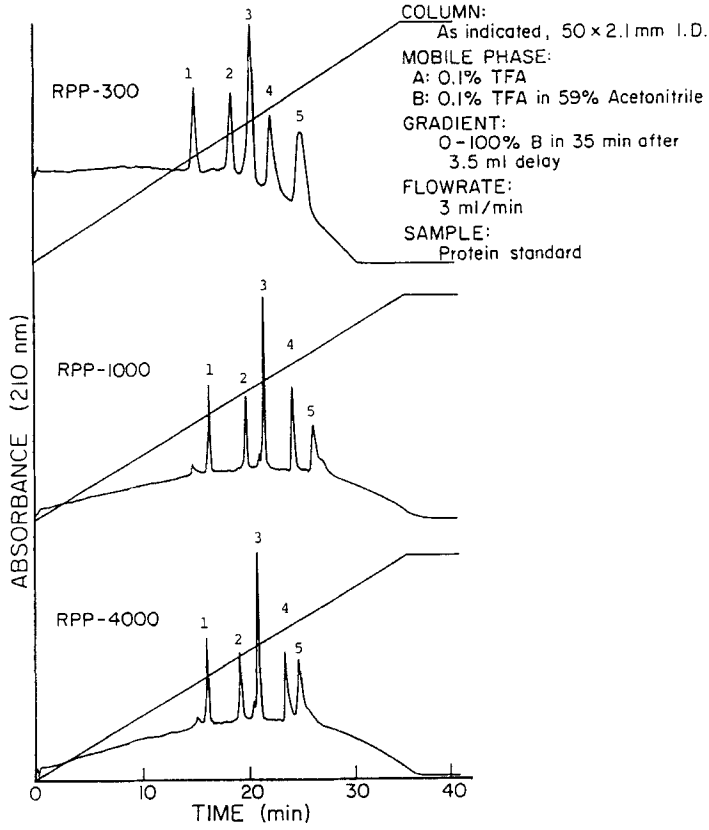
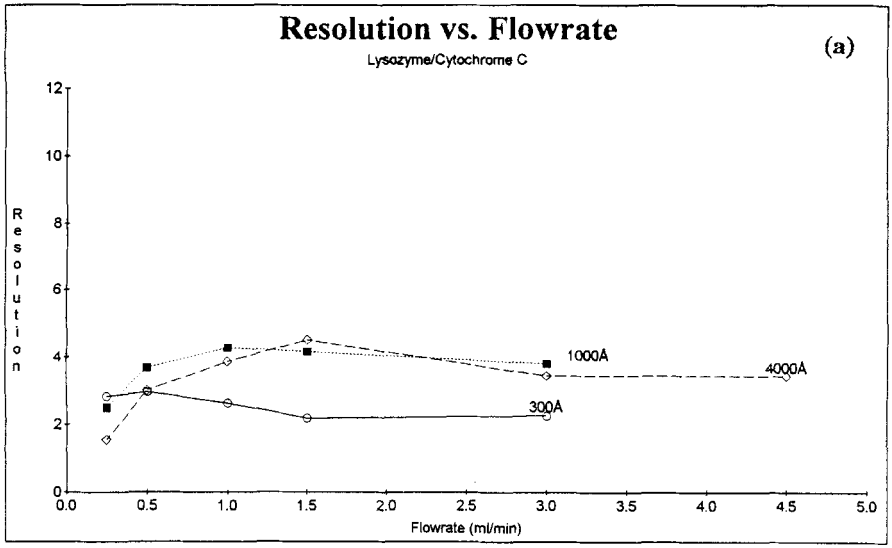


FIGURE 1. The effect of pore diameter on resolution of proteins. Columns: SynChropak RPP, RPP-1000 and RPP-4000, 50 x 2.1mm I.D. Flowrate:
3 ml/min. Gradient: 35 min gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 59% acetonitrile (delay volume is 3.5 ml). Sample:
1. ribonuclease A, 2. cytochrome c, 3. lysozyme, 4. transferrin and 5. bovine serum albumin. 1.2 µg of cytochrome c and 2.4 µg of each of the other proteins were injected.



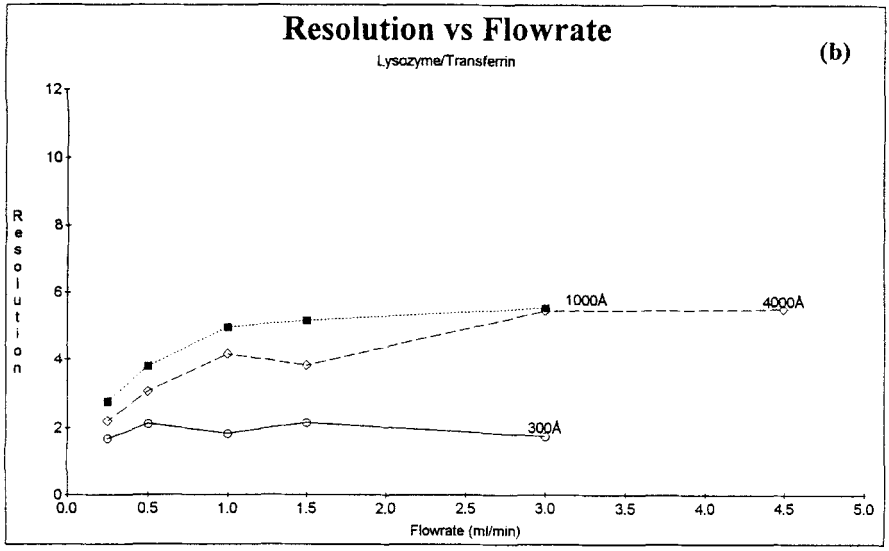


FIGURE 2. The effect of flowrate (linear velocity) on resolution of protein pairs: a. lysozyme/cytochrome c; b. lysozyme/transferrin; c. transferrin/BSA. Conditions as in Figure 1.

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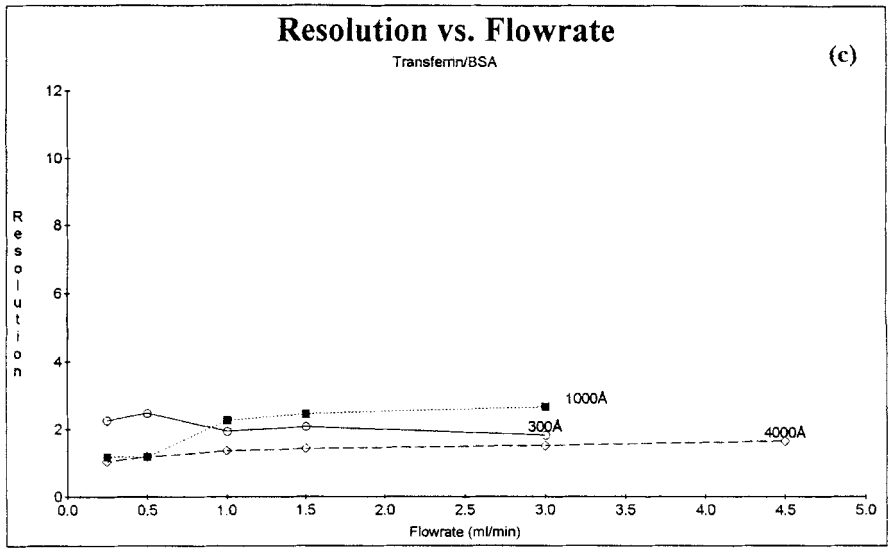

FIGURE 2 (Continued)

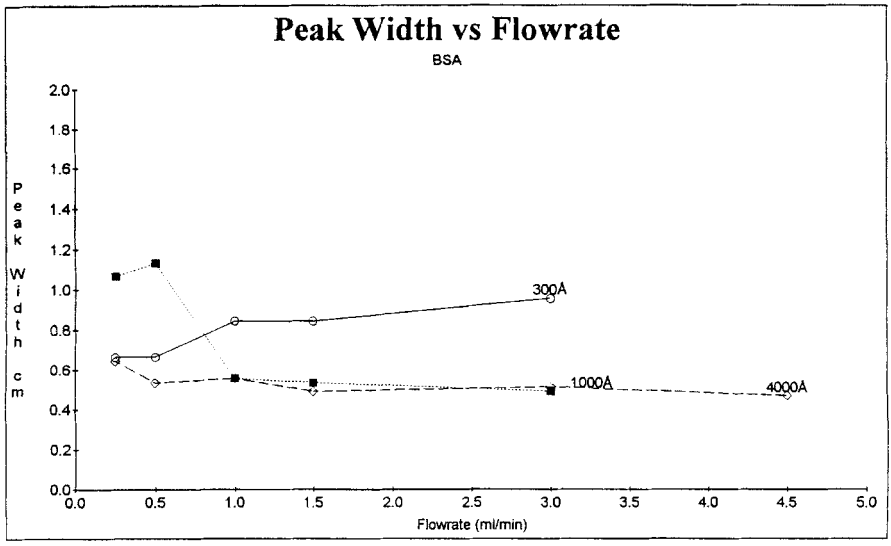

FIGURE 3. The effect of flowrate (linear velocity) on peak width for BSA. Conditions as in Figure 1.

peak widths. The lower retention on the 4000Å support is due to its lower surface area and larger particle diameter.

To verify these observations with another set of columns and an alternative gradient and mobile phase, a five minute methanol gradient at 3 ml/min was used. The peak widths of cytochrome c were the same on both 300Å and 4000Å supports, but the lysozyme and bovine serum albumin peaks were narrower on the 4000Å. Better resolution was again achieved on the 4000Å support than the 300Å despite the larger particle diameter of the former, as seen in Figure 4. Under these gradient conditions, some of the BSA peaks were totally resolved from the primary one so BSA did not look as broad.

Effect of Pore Diameter on Peptide Resolution

To test whether pore diameter would influence the effects of linear velocity on the resolution of small peptides, a decapeptide mixture was run in a parallel study. It can be seen in Figure 5 that the resolution of peptides was better on the 300Å and 1000Å supports, which are 7 μm, than on the 4000Å, which is 10μm. The graphs in Figure 6 confirm that at all flowrates the 300Å and 1000Å supports are superior to the 4000Å in terms of resolution of peptides.

Effect of Ligand Chain

The preceding studies utilized reversed phase columns with octadecyl (C-18) ligands. To investigate whether a shorter ligand chain might influence resolution on microbore columns run with high linear velocities, 300Å and 4000Å silica supports with C-4, C-8 and C-18 ligands were compared at 3 ml/min. Five minute gradients with methanol or isopropanol were implemented. There were few differences in selectivity,

FIGURE 4. The effect of pore diameter on protein resolution. Columns: SynChropak RPP and RPP-4000, 50 x 2.1mm I.D. Flowrate: 3 ml/min. Gradient: 5 min gradient from 0.1% trifluoroacetic acid in 10% methanol to 0.1% trifluoroacetic acid in 100% methanol. Sample: 1. ribonuclease A (75 µg),
2. cytochrome c (15 µg), 3. lysozyme (10 µg) and 4. bovine serum albumin
(40 µg).

resolution or retention among the chain lengths. For peptides, the C-8 and C-18 chains gave slightly better resolution. The two larger proteins gave somewhat higher band widths on the C-18 columns, confirming the general theory that short chain ligands are better for protein analysis. Generally, differences caused by ligand chain lengths were indistinguishable with these proteins and peptides. The more notable

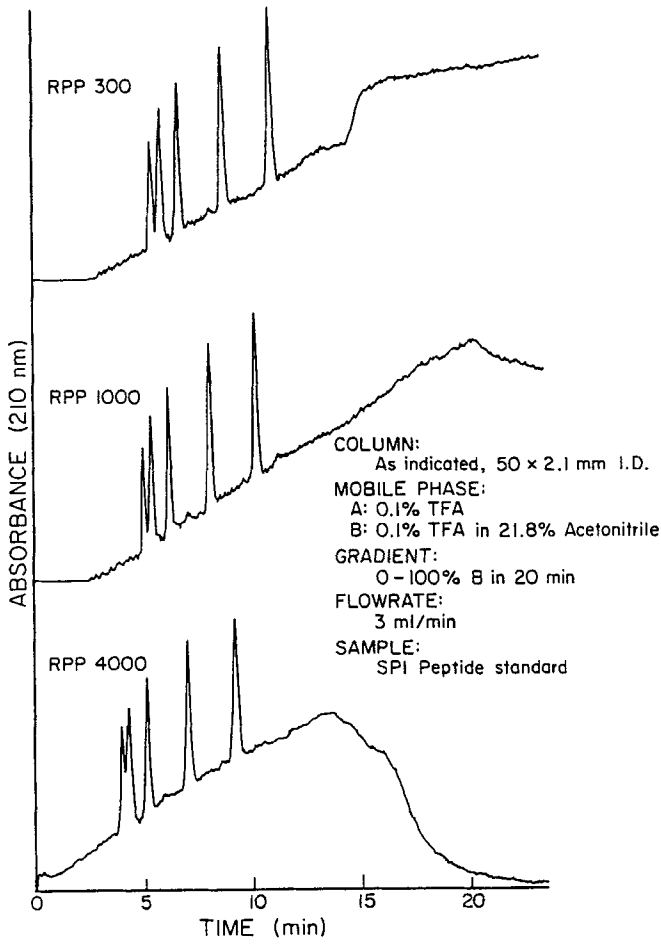

FIGURE 5. The effect of pore diameter on peptide resolution. Columns: SynChropak RPP, RPP-1000 and RPP-4000, 50 x 2.1mm I.D. Flowrate: 3 ml/min. Gradient: 20 min gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 21.8% acetonitrile. Sample: 2 µg SPI peptide standard.

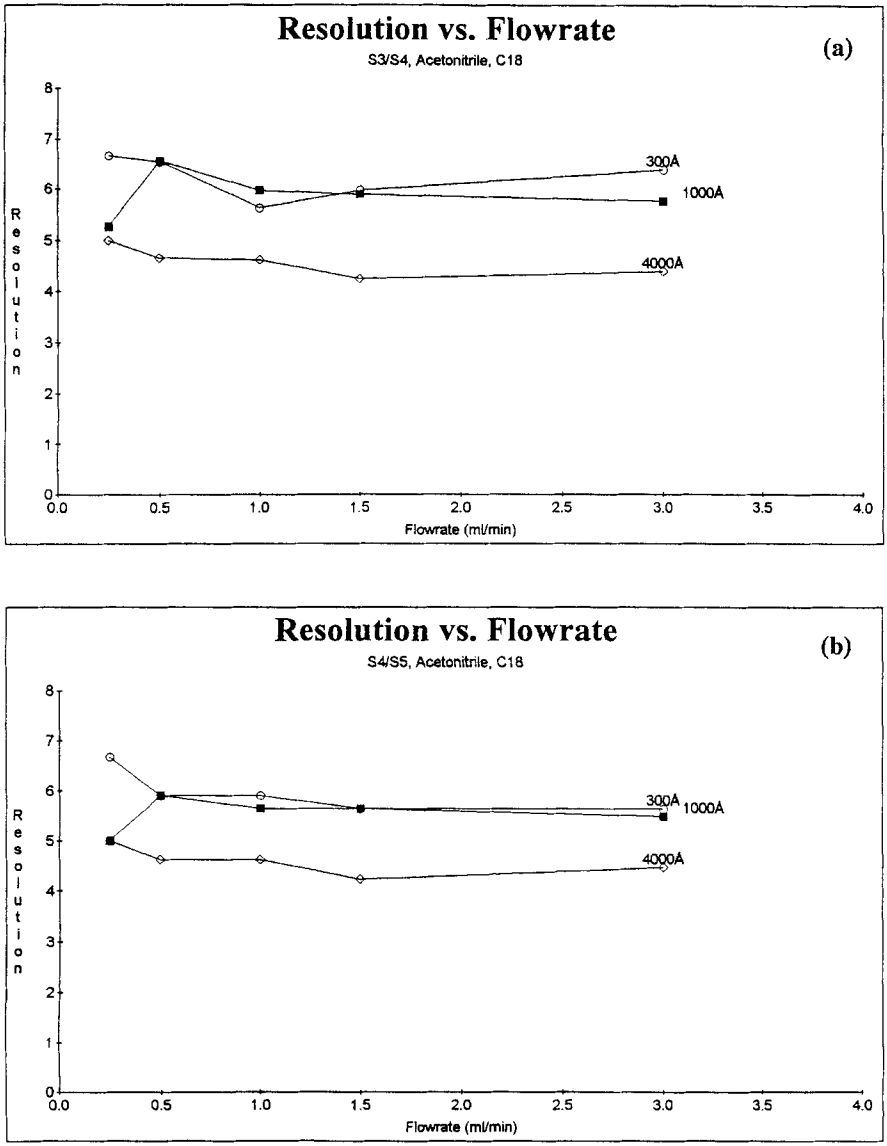

FIGURE 6. The effect of flowrate (linear velocity) on resolution of peptide pairs. Columns: SynChropak RPP, RPP-1000 and RPP-4000, 50 x 2.1mm I.D. Flowrate: 3 ml/min. Gradient: 20 min gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 21.8% acetonitrile. Sample: 2 µg SPI peptide standard.

differences were between the pore diameters. For all ligands, 4000Å gave superior resolution of proteins while 300Å was best for peptides.

Stability

To assess whether the porous supports used in this study could tolerate the harsh conditions of high linear velocities with trifluoroacetic acid in the mobile phase, a stability study was carried out using 0.1% trifluoroacetic acid at 3 ml/min on C-18 300Å and 4000Å columns. After each liter of throughput, the columns were tested with peptide standards. On both columns, retention times decreased no more than 5 - 8% from the initial run and peak widths remained relatively constant after 4 liters of solvent. This indicates that at least 25,000 column volumes or 250 runs of 5 min duration at 3 ml/min were not deleterious.

CONCLUSIONS

Microbore columns containing 1000Å or 4000Å reversed phase supports were effectively used at linear velocities of 15 - 20 mm/s to analyze proteins rapidly. At all flowrates except the slowest, the resolution of protein pairs not including BSA was better on 1000Å and 4000Å reversed phase columns than on 300Å. Resolution involving BSA was not as high on the 4000Å support because of its shorter retention and its heterogeneity.

Peptide resolution was best on the 300Å support under all conditions. The decapeptides would have total access to the surface of each of the 300Å, 1000Å and 4000Å supports. The lower resolution of the peptides on the 4000Å supports was partially due to the lower retention caused by the significantly lower surface area of the 4000Å support. The fact that the improvement of resolution with flowrate was observed for

proteins but not peptides would suggest that the band spreading was caused by the relative sizes of the solute and the pores. The pore diameter effects were confirmed when alternative chain lengths were used. Negligible differences on protein and peptide retention were seen between various ligands under the very short analysis conditions of this comparative study (3 ml/min, 5 min gradient).

The 1000Å and 4000Å silicas used in this study have some bimodal characteristics while the 300Å has a more uniform pore structure (18). The silica with an average pore diameter of 1000Å has sets of pores which are between 500Å and 3000Å (18). The 4000Å average pore diameter silica additionally contains some larger pores up to 5000Å (18). Mass transfer of proteins was facilitated by the presence of the large pores (> 500Å) which were absent in the 300Å supports. Some improvement of resolution with increased flowrate could be due to decreased contact time with the hydrophobic bonded phase, because contact of proteins with ligand chains sometimes results in unfolding or denaturation.

Both 300Å and 4000Å non-endcapped reversed phase supports held up well to the high flowrates and acidic conditions used in this study. Little degradation was seen after the equivalent of 250 gradient runs.

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