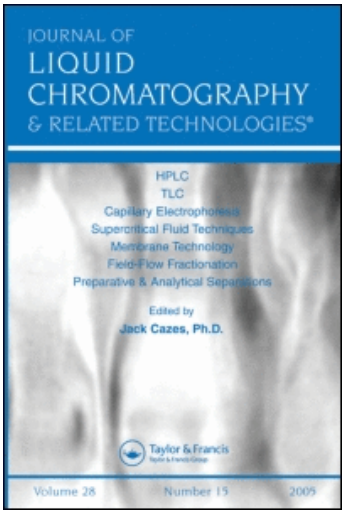

Journal of Liquid Chromatography & Related Technologies

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

Mechanistic Considerations on the Reversed Phase Liquid Chromato-Graphic Separation of Proteins

R. S. Blanquet[ab]; K. H. Bui[ac]; D. W. Armstrong[a]

[a] Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas [b] Department of Biology, Georgetown University, Washington, D. C. [c] ICI America, Biomedical Research Division, Wilmington, DE

To cite this Article Blanquet, R. S. , Bui, K. H. and Armstrong, D. W.(1986) 'Mechanistic Considerations on the Reversed Phase Liquid Chromato-Graphic Separation of Proteins', Journal of Liquid Chromatography & Related Technologies, 9: 9, 1933 — 1949

To link to this Article: DOI: 10.1080/01483918608078753

URL: http://dx.doi.org/10.1080/01483918608078753

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(9), 1933-1949 (1986)

MECHANISTIC CONSIDERATIONS ON THE REVERSED PHASE LIQUID CHROMATOGRAPHIC SEPARATION OF PROTEINS

R. S. Blanquet†, K. H. Bui+, and
D. W. Armstrong*
Department of Chemistry and Biochemistry
Texas Tech University
Lubbock, Texas 79409-4260

ABSTRACT

The effect of column length, stationary phase chemistry, flow rate and gradient variation and direction on the separation of seven proteins was evaluated. In general, resolution increased with shorter columns and higher flow rates. Nontraditional reverse gradients (from acetonitrile to buffer) sometimes produced better protein separations than traditional reversed phase gradients. Bonded phase chemistry has little effect on gradient

*Author to whom correspondence should be addressed.

†Current Address: Department of Biology, Georgetown University, Washington, D. C.

+Current Address: ICI America, Biomedical Research Division, Wilmington, DE

 0148-3919/86/0909-1933$3.50/0

protein separations, but silica gel properties (e.g., surface area, pore size, etc.) did. Both retention and resolution varied considerably with the nature of the gradient used.

INTRODUCTION

In the last few years, several papers and reviews have been published on the practice, theory and mechanism of protein separation by reversed phase liquid chromatography (1-30) and have contributed to the development of a useful technique. Many of the shortcomings, such as protein denaturation, have been considered as well (26, 28). Conversely, the theoretical and mechanistic aspects of this technique are not as advanced or well understood (31). There are several reasons for this state of affairs. The previously delineated theoretical problems stem from the mathematical difficulty in characterizing complex heteropolymers (31). Experimental problems and anomalies exist as well. In particular, the lack of consistent results between analogous columns, laboratories, etc. can sometimes be more pronounced for proteins than for small molecules.

In this work the effect of column length, stationary phase chemistry, flow rate, solvent composition and gradient control on protein separations are examined. In some cases our results are contrary to what has been previously reported and/or believed. These results tend to support the view that the reversed phase separations of proteins is not well understood from a mechanistic standpoint. However, considerable progress is being made as

researchers become less constrained by many of the traditional chromatographic concepts.

EXPERIMENTAL

Materials

Highest available purity ribonuclease, insulin, cytochrome C, lysozyme, bovine serum albumin, myoglobin and ovalbumin were obtained from Sigma. Trimethylchlorosilane, n-butyldimethylchlorosilane, n-octyldimethylchlorosilane, n-octadecyldimethylchlorosilane and diphenyldichlorosilane were obtained from Petrarch. Five micron average diameter silica with average pore sizes of 60 Å and 300 Å were obtained from Advanced Separation Technologies, Inc. (ASTEC) as were the stainless steel columns, frits and fittings. All columns were slurry packed (13,000 psi) by ASTEC. HPLC-grade solvents were obtained from Fisher.

Methods

All bonded phase packings were made under identical conditions. The silica gel was dried overnight at 170°C and subsequently slurried in anhydrous toluene. The reflux apparatus was fitted with a Dean Stark trap. The desired amount of a substituted chlorosilane was added and the slurry was refluxed for three hours. The product was isolated, washed and endcapped before being packed into columns. Both isocratic and gradient

separations were done at room temperature with a Shimadzu Model LC-4A liquid chromatograph. The variable wavelength detector contained a 13 µl flow cell and was set at 280 nm. The aqueous phase consisted of 0.1% trifluoroacetic acid (TFA). All protein solutions were 1 µg/ml concentration in 0.1% TFA. Flow rates and injection volumes are reported in the legends of the individual figures and tables.

RESULTS AND DISCUSSION

Column length is considered an important parameter in most chromatographic separations. This is also true for the RPLC of proteins, but not necessarily for the same reasons as in conventional small molecule separations. Increasing column length is one possible way of increasing the number of theoretical plates for solutes that can be treated via conventional LC theory. However, qualitative reports have indicated that short columns provide about the same resolution (for proteins) as longer columns (13,14). The only definitive mechanistic work in this area was done for synthetic polymers and this cannot be applied directly to proteins (32). The effect of column length on the retention and resolution of cytochrome c and bovine serum albumin with gradient separation is shown in Table 1. The retention of the proteins increased somewhat with column length. However, their resolution decreased with column length. This result was a consequence of lower efficiency with longer columns as well as the fact that the peak to peak separation is less for longer columns (Table 1). It

was found that the relationship between column length and peak to peak separation could vary with the proteins studied. In comparing the protein results to those for the gradient separation of synthetic polymers (32) there are similarities and differences. Specifically, resolution also decreased for synthetic polymers when longer columns were used (31,32). However, the retention times of synthetic polymers did not significantly increase with column length as did those of the proteins (Table 1). It is interesting to consider the minimum and/or optimum length for a reversed phase column in protein separations. In fact, it is not the length, per se, but the amount of packing that is important. There is a minimum amount of stationary phase needed to immobilize the injected protein and prevent "breakthrough" of excess solute in the void volume. However, the absolute amount of stationary phase needed can vary considerably with the separation conditions. Factors such as the amount and nature of the protein(s) injected, mobile phase composition, flow rate, column geometry, capacity and surface area of the stationary phase, size of the stationary phase and so on, all affect the minimum amount of packing required. Consequently a small amount of packing might work well for one separation but be inadequate for another. In this study, columns between 3 and 5 cm in length and 0.46 cm diameter gave good resolution and no "breakthrough" regardless of the separation conditions employed. Shorter columns, particularly < 2 cm, also gave excellent results, however, protein "breakthrough" was difficult to control in many cases. Thus, a good general reversed

Table 1. Effect of column length on separation of cytochrome C and bovine serum albumin (BSA). Column: Astec diphenyl 300Å, Mobile phase: (A)-triethylamine acetate buffer, pH, 4.0, (B)-n-propanol. Gradient rate: 1.5% min^{-1}. Flow rate: 0.5 ml min^{-1}.

Column length (cm)	Cyto C (k_1)	BSA (k_2)	α	R
5	8.70	14.83	1.70	0.74
10	10.29	15.83	1.49	0.70
15	13.33	16.32	1.23	0.65
20	19.54	22.55	1.15	0.57

phase column for analytical protein separations should contain sufficient packing to interact with all of the injected solute but not be excessively long so that resolution suffers.

An additional advantage of shorter columns for many protein separations is that the recoveries are often higher and smaller samples can be analyzed. Extrusion of the column packings in this study and treatment with ninhydrin indicated that large amounts of protein were irreversibly bound to the stationary phase in all cases. Reversed phase TLC of the seven proteins in this study (see Experimental Section) indicated that complete elution could only be approached after the stationary phase had been previously coated (or saturated) with sample. Thus in many cases it is

Table 2. Effect of stationary phase on protein separation. Peak: 2-Ribonuclease A, 3-Insulin, 4-Cytochrome C, 5-Lysozyme, 6-Bovine serum albumin, 7-Myoglobin, 8-Ovalbumin. Flow rate: 1.0 ml min^{-1}. Mobile Phase: A- 0.1% TFA in water, B- 0.1% TFA in acetonitrile. Gradient: 10-100% acetonitrile in 45 minutes.

Stationary Phase (300Å)	Peak Retention Time (min)						
	2	3	4	5	6	7	8
C-1	13.3	14.0	15.5	18.0	18.5	20.8	27.5
C-4	12.5	14.5	16.3	17.8	20.5	23.3	28.8
C-8	11.0	13.5	15.3	17.0	19.0	22.0	27.0
C-18	13.5	15.0	16.3	17.8	20.3	22.0	30.0
Diphenyl	12.3	14.5	16.0	17.5	19.6	22.5	29.3

logical to use a smaller column in which less sample is lost via preliminary coating of the stationary phase.

Given the fact that most silica gel based reversed phase columns used for protein separations are coated with proteinatious material, it is interesting to consider the effect of the stationary phase chemistry on protein separations. Table 2 shows the effect of five different bonded reversed phase packings (on identical 300 Å silica gel) on the retention of seven different proteins. It is apparent that neither the selectivity nor the

retention of any protein is significantly affected by the nature of the bonded phase under these conditions.

The flow rate has been shown to have an effect on the separation of proteins (10,21). Earlier investigations suggested that optimal resolution of proteins occurs at lower flow rates (10). Figure 1 shows the effect of flow rate on the separation of seven proteins using fixed gradient elution. Contrary to what was previously reported, higher flow rates tended to decrease retention times and increase resolution under the conditions indicated in Figure 1. It was apparent from this particular data that the best separation occured at the highest flow rate.

Perhaps the most interesting mechanistic aspect of protein separations by reversed phase LC involves the effect of solvent compositions and gradient type on retention. For the purpose of this discussion, the denatured protein peaks (which were often present) will not be considered. Cohen et al. (28) recently considered and discussed the appearance of denatured protein peaks in reversed phase LC. It is well known that plots of retention (isocratic) versus solvent composition have minima for proteins, peptides and occasionally, other compounds (20,31). Typical plots for myoglobin, ribonuclease and insulin are shown in Figure 2. Several interesting conclusions can be drawn from such plots. First elution will only occur in certain solvent composition "windows" and these "windows" vary with each protein. Traditional LC gradients begin with mobile phases consisting of a high percentage of aqueous buffer and increase the volume percent of

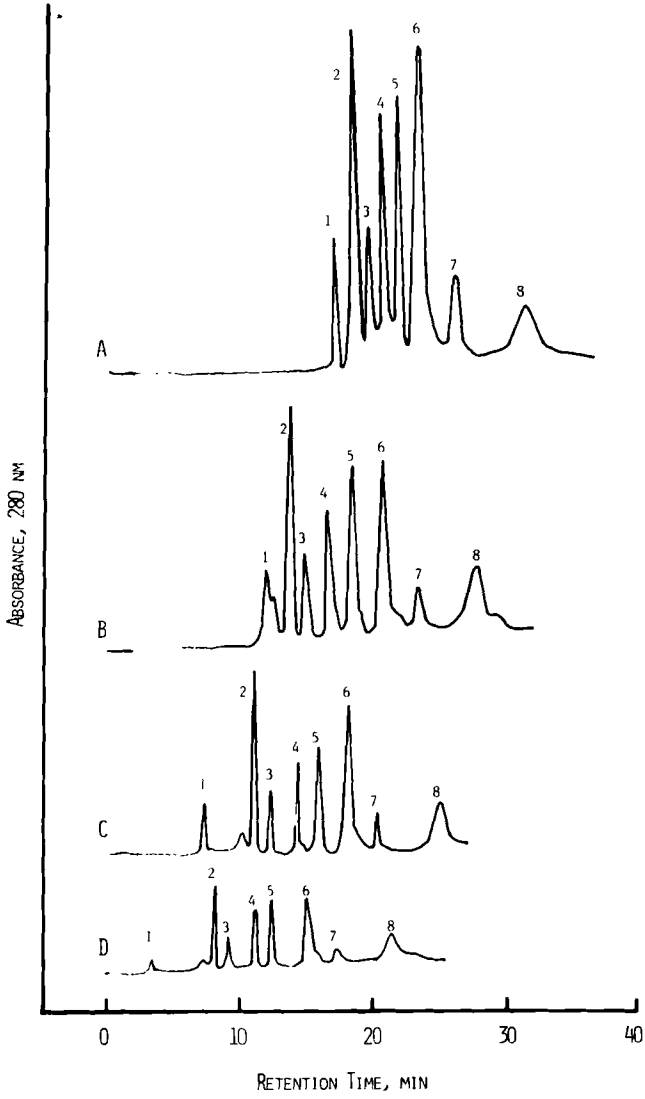

Figure I. Effect of flow rate on protein separation. Peak: 1-impurities, 2-ribonuclease A, 3-insulin, 4-cytochrome c, 5-lysozyme, 6-bovine serum albumin, 7 myoglobin, 8-ovalbumin. Flow rate: A- 0.5 ml min^{-1}, B- 1.0 ml min^{-1}, C- 2.0 ml min^{-1}, D- 4.0 ml min^{-1}. Column: Astec C-8 (50 x 4.6 mm), 300Å. Mobile phase: A = 0.1% TFA in water, B = 0.1% TFA in acetonitrile. Gradient: 10-100% in 45 minutes.

organic modifier with time. It is apparent from Figure 2 that little or no protein elution will occur with mobile phases containing less than 30% acetonitrile by volume (on this particular column). As the acetonitrile concentration is increased proteins begin to elute and their elution order is governed by the relative position or order of the left hand side of the curves in Figure 2. Thus, according to Figure 2, ribonuclease would be expected to elute first, insulin second and myoglobin last when running a buffer to acetonitrile gradient. It is also possible to run a nontraditional acetonitrile to buffer gradient. According to Figure 2, this type of gradient would also produce a separation. In this case the protein elution order would be governed by the relative position of the right hand portion of the curves in Figure 2. Furthermore the elution order should be exactly opposite that for the traditional gradient (see Figure 2). Experimental verification of the reverse in retention behaviour for opposite gradients is given in Table 3. It also should be noted that the nontraditional gradient B (acetonitrile —> buffer) produced a far superior separation of these particular proteins. The peaks were much sharper and the separation was complete in almost half the time. It is now thought that the increase in retention of proteins and peptides at high organic modifier concentrations (Figure 2) is a solubility based phenomenom (31). It should be noted, however, that this is only true of the water soluble proteins. In the case of lipophilic membrane proteins, the organic modifier may very well

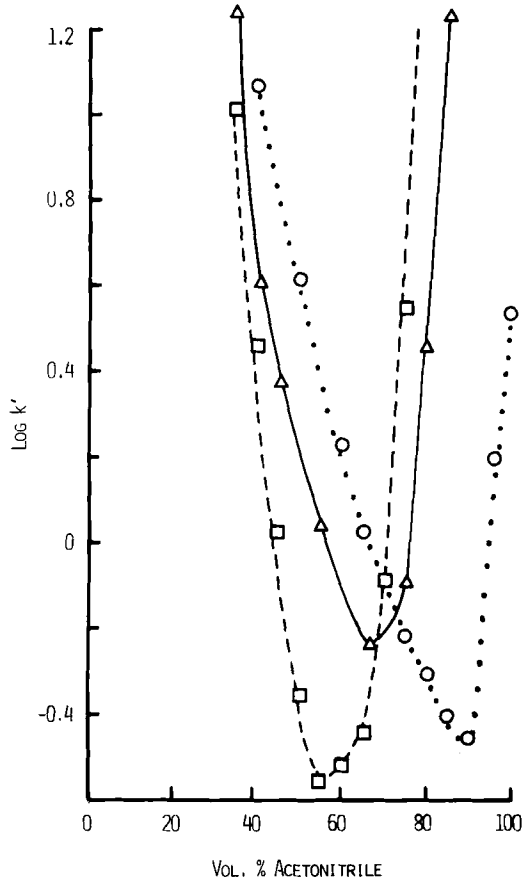

Figure II. Plots showing the effect of solvent composition on isocratic protein retention for: ribonuclease ([]), insulin (Δ) and myoglobin (O.) Column: Astec C-8 (50 x 4.6 mm), 60Å. Buffer: 1% trifluoroacetic acid.

Table 3. The effect of gradient reversal on protein elution sequence. Gradient A; 5-100% acetonitrile in 30 min. Gradient B; 90-5% acetonitrile in 30 min. Column Astec C-8, 60 Å

Protein	Retention Time (T_R)	
	Gradient A	Gradient B
Ribonuclease	17.63	13.03
Insulin	18.26	10.52
Myoglobin	21.43	1.91

be a good solvent and water or buffer a poor solvent. Retention profiles for these proteins would be expected to be very different from those described in this report.

One can manipulate the separation of proteins by adjusting the gradient. The most obvious way is by reversing the gradient as in the previous discussion and Table 3. However, one can also exercise considerable control by optimizing the rate of gradient change and the starting mobile phase composition. Table 4 shows the effect of gradient rate on retention. In this case, the slower gradient produced longer retention and a better separation for all seven proteins. Tables 5 and 6 show the effect of the starting mobile phase composition and protein loading on retention and resolution. As would be expected from the curves in Figure 2, starting the gradient at 35% acetonitrile (Table 6) produces much

Table 4. Effect of gradient rate on protein separation. Peak: 1-Impurities, 2-Ribonuclease A, 3-Insulin, 4-Cytochrome C, 5-Lysozyme, 6-Bovine serum albumin, 7-Myoglobin, 8-Ovalbumin. Gradient: 10-90% Acetonitrile. Flow rate: 4.0 ml min^{-1}. Column: Astec C-8 (50 x 4.6 mm), 300Å.

Gradient Time Span (min)	Peak Retention Time (min)							
	1	2	3	4	5	6	7	8
8	2.7	3.1	3.4	3.7	4.0	4.4	4.9	5.8
16	3.1	4.4	5.0	5.7	6.3	7.7	8.1	9.9
24	3.3	5.9	6.5	7.7	8.5	9.7	11.0	13.7

Table 5. Effects of protein loading on separation of insulin and ribonuclease. Gradient: 10-90% acetonitrile in 30 min. Column: Astec C-8, 300Å. Flow rate: 0.5 ml min^{-1}. Protein concentrations: 1 µg/ml.

Conc (µl)	Insulin (T_R)	RNase (T_R)	T_R	R
10	15.31	14.37	0.95	0.39
50	15.10	13.96	1.13	0.40
100	14.97	13.86	1.11	0.38
150	14.70	13.56	1.14	0.40
190	14.87	13.67	1.21	0.38

Table 6. Effects of protein loading on separation of insulin and ribonuclease. Gradient: 35-70% acetonitrile in 30 min. Column: Astec C-8, 300Å. Flow rate: 0.5 ml min^{-1}.

Conc (µl)	Insulin (T_R)	RNase (T_R)	T_R	R
10	5.39	3.80	1.59	0.42
50	4.92	3.60	1.30	0.34
100	4.80	3.77	1.03	0.27
150	4.76	3.87	0.89	0.23
200	4.87	4.02	0.85	0.23

lower retention times than an analogous separation starting at 10% acetonitrile (Table 5). The starting solvent composition did not affect protein resolution when small amounts of sample were injected. Injection of large samples produced slightly lower retention times but little change in resolution for separations starting at 10% acetonitrile (Table 5). However, when the gradient was started at 35% acetonitrile (Table 6) the resolution tended to decrease with increasing sample size.

It is apparent that the reversed phase LC separation of proteins is a very complex phenomenom. Denaturation and sample loss further complicate an already muddled mechanistic picture. Much of modern LC dogma does not apply to the reversed phase separation of proteins (31). Recently derived polymer chromatographic theory has produced a few possible explanations

but it also fails (in its ideal form) to predict and describe much of the protein data (supra vide). We believe that the best future approach is to work with simpler molecules (that can be mathematically modeled) which produce retention curves analogous to those in Figure 2. These studies are currently underway.

ACKNOWLEDGEMENT

Support of this work by the Department of Energy, Office of Basic Energy Science (DE-AS0584ER13159) is gratefully acknowledged.

REFERENCES

1. Rivier, J. E., J. Liq. Chromatogr. 1, 343 (1978).

2. Rubinstein, M., Rubinstein, S., Familletti, P. C., Miller, R. S., Waldman, A. A. and Pestku, S. Proc. Natl. Acad. Sci. USA 76, 640 (1979).

3. Rubinstein, M., Chen-Kiang, S., Stein, S. and Udenfriend, S. Anal. Biochem. 95, 117 (1979).

4. Hearn, M. T. W., Grego, B., Hancock, W. S. J. Chromatogr. 185, 429 (1979).

5. Rubinstein, M. Anal. Biochem. 98, 1 (1979).

6. Van Oss, C. J., Absolom, D. R. and Newmann, A. W. Sep. Sci. Technol. 14, 305 (1979).

7. O'Hare, M. J., Nice, E. C. J. Chromatogr. 171, 209 (1979).

8. Hearn, M. T. W. and Hancock, W. S. J. Chromatogr. Sci. 18, 288 (1980).

9. Jennissen, H. P. Protides Biol. Fluids, Proc. Colloq. 27, 765 (1980).

10. Lewis, R. V., Fallon, A., Stein, S., Gibson, K. D. and Udenfriend, S. Anal. Biochem. 104, 153 (1980).

11. Regnier, F. E. and Gooding, K. M. Anal. Chem. 103, 1 (1980).

12. Pearson, J. D., Mahoney, W. C., Hermodson, M. A. and Regnier, F. E. J. Chromatogr. 207, 325 (1981).

13. Pearson, J. D., Lin, N. T. and Regnier, F. E. Anal. Biochem. 124, 217 (1982).

14. Hearn, M. T. W., Regnier, F. E. and Wehr, C. T. Proceedings of the First International Symposium on HPLC of Proteins and Peptides. Academic Press, New York, 1983.

15. Grego, B. and Hearn, M. T. W. Chromatographia 14, 589 (1981).

16. Hearn, M. T. W. and Grego, B. J. Chromatogr. 218, 497 (1981).

17. Kloas, E. B., Horvàth, Cs., Melander, W. R. and Nahum, A. J. Chromatogr. 203, 65 (1981).

18. Barford, R. A., Sliwinski, B. J., Breyer, A. C. and Rothbart, H. L. J. Chromatogr. 235, 281 (1982).

19. Burgess, A. W., Knesel, J., Sparrow, L. G., Nicola, N. A. and Nice, E. C. Proc. Natl. Acad. Sci. USA 79, 5753 (1982).

20. Hearn, M. T. W. Advances in Chromatography, Vol. 20, J. C. Giddings et al., eds. Marcel Dekker, New York, 1982, pp. 2-82.

21. Cooke, N. H. C., Archer, B. G., O'Hare, M. J., Nice, E. C., Capp, S. L. J. Chromatogr. 255, 115 (1983).

22. Hearn, M. T. W. and Grego, B. J. Chromatogr. 255, 125 (1983).

23. Hearn, M. T. W. High-Performance Liquid Chromatography, Vol. 3, Cs. Horvàth, ed. Academic Press, New York, 1983, pp. 87-155.

24. Scoble, H. A. and Brown, P. R. High-Performance Liquid Chromatography, Vol. 3. Cs. Horvàth, ed. Academic Press, New York, 1983, pp. 1-47.

25. Hancock, W. S. and Sparrow, J. T. High-Performance Liquid Chromatography, Vol. 3, Cs. Horvàth, ed. Academic Press, New York, 1983, pp. 49-85.

26. Trumbore, C. N., Tremblay, R. D., Pensrose, J. T., Mercer, M. and Kelleher, F. M. J. Chromatogr. 280, 43 (1983).

27. Sasagawa, T., Ericsson, L. H., Teller, D. C., Titani, K. and Walsh, K. A. J. Chromatogr. 307, 29 (1984).

28. Cohen, S. A., Benedek, K. P., Dong, S., Tapuki, Y., Karger, B. L. Anal. Chem. 56, 217 (1984).

29. Stadalius, M. A., Gold, H. S., Snyder, L. R. J. Chromatogr. 296, 31 (1984).

30. McLeod, A., Wood, S. P. J. Chromatogr. 285, 319 (1984).

31. Armstrong, D. W. and Boehm, R. E. J. Chromatogr. Sci. 22, 378 (1984).

32. Bui, K. H., Armstrong, D. W. and Boehm, R. E. J. Chromatogr. 288, 15 (1984).