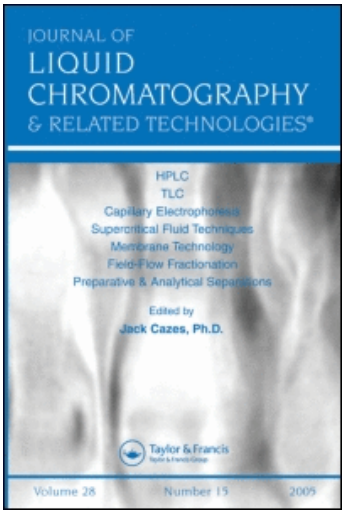

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Reverse-Phase HPLC of Proteins: Effects of Various Bonded Phases

Randolph V. Lewis[a]; Daryll Dewald[a]
[a] Department of Biochemistry, University of Wyoming University Station, Laramie, Wyoming

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REVERSE-PHASE HPLC OF PROTEINS: EFFECTS OF VARIOUS BONDED PHASES

Randolph V. Lewis and Daryll DeWald
Department of Biochemistry
University of Wyoming
University Station, Box 3944
Laramie, Wyoming 82071

ABSTRACT

A variety of organic phases bonded to silica can be used effectively for the reverse-phase high performance liquid chromatography of large proteins. These include octyl, octadecyl, cyanopropyl and diphenyl bonded phases. The differences in retention characteristics and selectivity among these bonded phases is demonstrated with several standard proteins.

INTRODUCTION

The use of reverse-phase high performance liquid chromatography (hplc) for the separation and purification of peptides and small proteins has gained wide acceptance (1). This same methodology for large proteins has been used in a few instances (2,3) and shows promise for much wider use. The best reverse-phase chromatography conditions for large proteins utilize large pore silica supports (30-50nm pores) and slow flow rates

(2,4). These are necessary due to the size of the larger proteins and their slow diffusion rates. Although a variety of organic bonded phases have been used for the isolation of peptides and small proteins (5,6,7), an examination of various organic bonded phases for the reverse-phase chromatography of large proteins has not been reported. In this paper we demonstrate that octyl, octadecyl, diphenyl, and cyanopropyl bonded phases can be used effectively in the reverse-phase hplc of large proteins. In addition, the differences in selectivity with these different bonded phases is described.

MATERIALS AND METHODS

Collagen (M.W. 100,000), β-lactoglobulin-B (M.W. 35,000), phosphorylase-B (M.W. 94,000), and human serum albumin (M.W. 68,000) were all purchased from Sigma. Pyridine, formic acid, propanol, and acetone (all from Baker Chemical Co.) were distilled over ninhydrin prior to use. The hplc system used was a Spectra-Physics 8700 equipped with a mixing chamber. The post-column fluorescamine detection system has been described previously (8). The chromatography buffers were 0.5M formic acid brought to pH 4.0 with pyridine and the same buffer containing 60% (v/v) 1-propanol. A flow rate of 0.75mL/min was used for all the chromatographies. The columns (4.6 X 250mm) were 'Bakerbond' Wide-Pore columns (Baker Chemical Co.) with 33nm pore size. Further details are provided in the figure legends.

RESULTS AND DISCUSSION

The advantage of using 33nm pore supports instead of 10nm pore supports is demonstrated in Fig. 1. The 33nm pore octyl column gives better separation of the standard proteins and

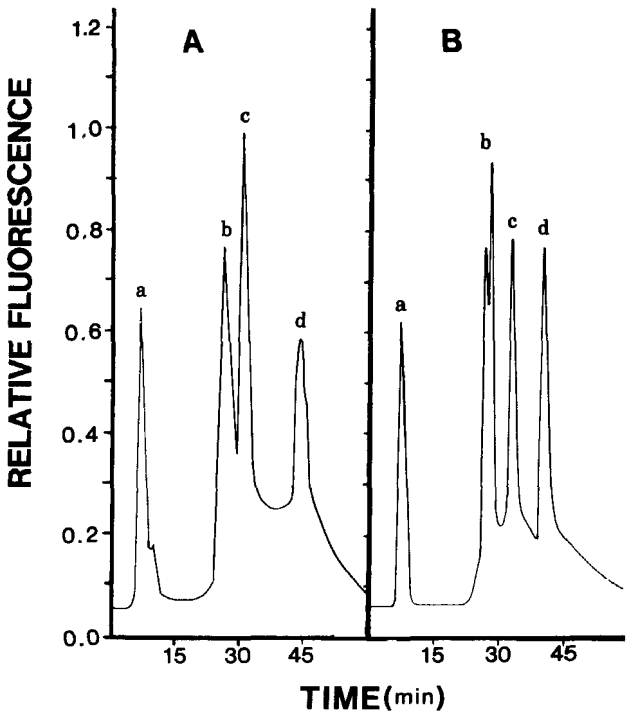

FIGURE 1. Comparison of 10nm and 33nm pore octyl columns. Phosphorylase B (10 μg), collagen (35 μg), human serum albumin (7 μg) and lactoglobulin B (10 μg) were dissolved in 1 mL of starting buffer for injection. This is the elution order of these proteins (identified a-d in the figure). The gradient was 1-propanol, 0-24% in 15 min, 24-48% in 55 min in 0.5M formic acid/0.4M pyridine pH 4.0 with a flow rate of 0.75 mL/min. Panel A is a Lichrosorb RP-8 column (10μ particles with 10nm pores). Panel B is a "Bakerbond" Wide Pore Octyl column (10 particles with 33nm pores).

partially separates the α_1 and α_2 chains of collagen which are unresolved on the 10nm pore octyl column. β-lactoglobulin-B elutes later from the small pore column and shows a small shoulder not seen on the large pore column. Thus, it appears that this protein is small enough to effectively penetrate the smaller pores. Although other factors may be influencing the differences between these columns none of the 10nm pore supports we have tested give resolution comparable to the 33nm pore supports.

In an effort to determine the effect that the various bonded phases would have on the elution of these proteins, identical amounts of each protein were chromatographed on each of the four types of columns using identical gradients (Fig. 2). From this figure it is clear that all four bonded phases produce excellent resolution. The gradient used was designed to separate these proteins on the octyl column. The protein elution times for each of the columns is shown in Table 1. Phosphorylase-B is only weakly retained (about 1 min beyond wash through) on all these columns. The columns all show good reproducibility and recoveries of the proteins ranged from 80-95%.

The octyl and octadecyl columns show very similar elution profiles and elution times (Fig. 2A and B, Table 1), although the collagen chains are separated to a slightly greater extent on the octadecyl support. This similarity is somewhat surprising in view of the large difference in hydrophobicity between the two bonded phases. This leads us to suggest that the octadecyl chain may be

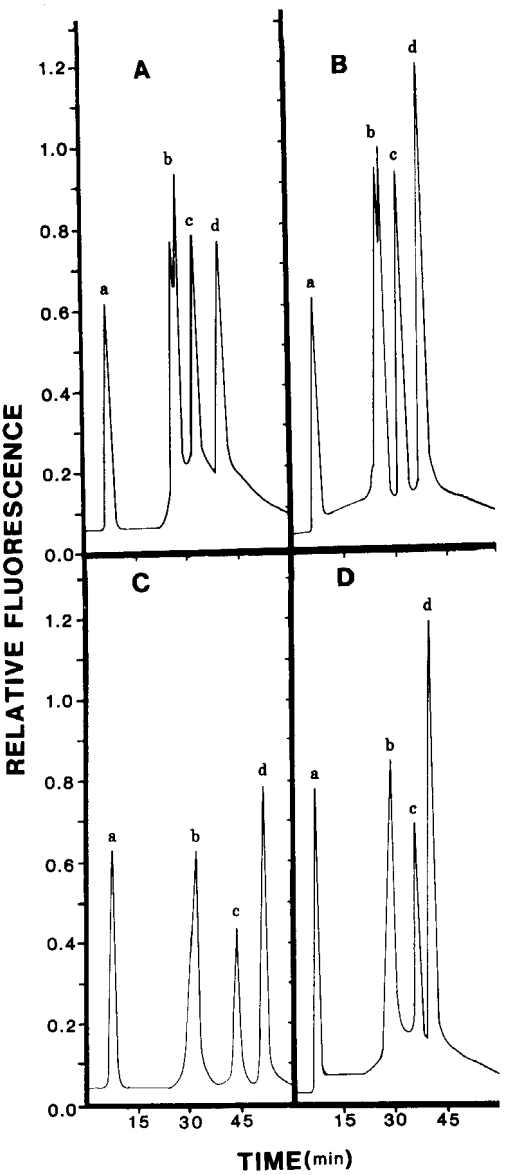

FIGURE 2. Elution from different bonded phases. Proteins and gradient used were the same as in Fig. 1. The columns were all "Bakerbond" Wide Pore columns. a) Octyl, b) Octadecyl, c) Diphenyl, and d) Cyanopropyl. Protein elution order is the same as Fig. 1.

TABLE 1

COLUMN		ELUTION TIME (Min)		
	PB	CL	HSA	LGB
Octyl (10nm)[2]	7	26.5 (0.5)	31.5 (0.3)	45 (0.3)
Octyl[3]	7	27,28 (0.3)	34 (0.4)	41 (0.4)
Octadecyl[3]	7	27,28 (0.3)	33 (0.3)	38.5 (0.4)
Cyanopropyl[3]	7	29.5 (0.2)	36.5 (0.4)	41 (0.4)
Diphenyl[3]	7	32.5 (0.1)	45.3 (0.3)	53.5 (0.4)

[1]The times are averages of at least 3 chromatographies with the S.E.M. in parentheses.
[2]E.M. Merck Lichrosorb octyl column (10μ particles).
[3]"Bakerbond" Wide Pore columns (33nm pores and 10μ particles). All columns were 4.6 x 250nm.
[4]The peaks are phosphorylase B (PB), collagen (CL), human serum albumin (HSA), and β-lactoglobulin B (LGB).

folding back on itself under the aqueous conditions employed. Another possible explanation is that the proteins we used have no hydrophobic sites large enough to discriminate between the two different chain lengths. Lower ionic strengths or different buffer systems might be used to accentuate differences between these two bonded phases.

The cyano-propyl column shows a slightly different elution profile than the two aliphatic carbon chain bonded phases (Fig. 2D). There is no resolution of the collagen chains with this bonded phase. However, their elution times, as well as that of human serum albumin are later relative to the octyl column (1.5 and 2.5 min respectively). In contrast, β-lactoglobulin-B elutes at the same time from both bonded phases. This selectivity difference between the cyano-propyl column and the aliphatic chain columns, indicates these differences can be exploited for protein

purifications. The cyano-propyl bonded phase has been shown to be effective in separating small proteins that differ in glycosylation (9). These large pore cyano-propyl columns may also be useful in this type of application with large proteins.

Retention times of the standard proteins on the diphenyl bonded phase are significantly different than with the other three bonded phases (Fig. 2C). The three retained proteins, collagen, human serum albumin, and β-lactoglobulin-B, elute much later from this column, the latter two proteins by 11 and 13 min respectively. From this data and data obtained using small pore columns with peptides (6) it is clear that the diphenyl bonded phase is interacting with proteins in a different manner than the straight chain bonded phases. It is likely that this interaction involves a combination of hydrophobic and aromatic stacking effects. Since increased retention times are not seen on a phenyl column (unpublished results) it appears the diphenyl group is required to obtain stacking with aromatic residues on the proteins. The smaller effect seen with collagen may be due to the relatively low percentage of aromatic groups in that protein.

From the results obtained in these studies it is evident that larger proteins (M.W. 50,000) can be effectively separated by reverse-phase hplc. As has been demonstrated for peptides, the use of different organic bonded phases greatly facilitates purifications by taking advantage of the differences in selectivity among the bonded phases. The proper gradient

conditions are very similar for all these bonded phases as was demonstrated in Fig. 2 where the same gradient was used for all four bonded phases. Thus it is possible to take advantage of the differing elution characteristics and selectivity provided by the different bonded phases without a great deal of trial and error to determine the appropriate buffer and organic modifier conditions.

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