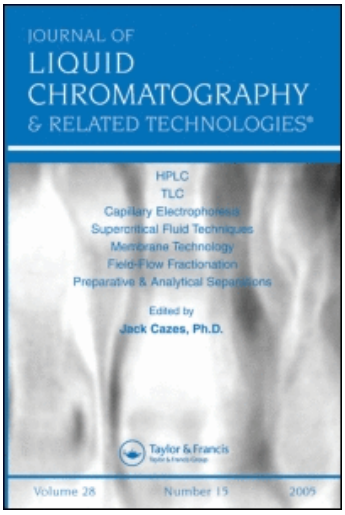

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The Influence of Reversed-Phase n-Alkyl Chain Length on Protein Retention, Resolution and Recovery: Implications for Preparative HPLC

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THE INFLUENCE OF REVERSED-PHASE n-ALKYL CHAIN LENGTH ON PROTEIN RETENTION, RESOLUTION, AND RECOVERY: IMPLICATIONS FOR PREPARATIVE HPLC.

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ABSTRACT

High-performance liquid chromatography (HPLC) of proteins on reversed-phase columns of varying n-alkyl chain length (C_2 to C_{22}) was studied using a trifluoroacetic acid/2-propanol mobile phase system. Protein resolution was influenced by chain length but retention times for proteins, unlike those of small molecules, were relatively constant, independent of chain length or carbon loading. Loading capacities were found to be affected by chain length, and aspects of protein interaction with stationary phase are discussed.

INTRODUCTION

High-performance reversed-phase liquid chromatography (RPLC)[1] has recently been widely adapted to peptide and protein separations. Since the first applications for proteins (1,2) two significant practices have enhanced RPLC utility tremendously; the use of trifluoroacetic acid (TFA) in mobile phases and the use of macroporous silica supports. TFA (3-5) and some other perfluorocarboxylic acids (6) were found to be excellent ion-pairing

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agents. Additional advantages of TFA over many other ion-pairing agents include UV transparency at 220nm (4), greater protein recovery (7), and easy removal of the eluant by lyophilization (b.p.72.4°C). Macroporous silica has been known for years to have high binding capacity (8) and excellent resolution (9) in high-performance anion-exchange chromatography of proteins, but it was not until the application to reversed-phase supports (10) that the utility of macroporosity was widely recognized.

It was recently reported that short n-alkyl chains ($\leq C_6$) further enhance protein recovery and resolution while sharply deminishing the "ghost peaking" effect common to C_8 and C_{18} chains (11). This communication extends those studies. Normally load capacity increases with n-alkyl chain length and stationary phase carbon content (12), rendering short n-alkyl chains undesirable for the preparative mode. Since previous loading studies were done with small solutes (<1000 daltons) and since proteins are known to interact with n-alkyl chains differently (13) than smaller molecules, the relationship of n-alkyl chain length or stationary phase carbon content to column load capacity was re-addressed. Short columns (5 cm) with n-alkyl chains ranging in length from C_2 to C_{22} were prepared and protein capacity, retention, and resolution in the analytical mode determined. It was found that protein load versus n-alkyl chain length differed substantially from values one would expect for small molecules. In addition, protein retention times were not found to increase with increasing alkyl chain length.

MATERIALS AND METHODS

Chemicals

n-Alkylchlorosilanes were purchased from Petrarch Systems (Levittown, PA). Vydac silica was obtained from The Separations Group (Hesperia, CA). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL). 2-Propanol was purchased from Fisher Scientific Company (Fair Lawn, NJ). Acetonitrile was obtained from J. T. Baker Chemical Company (Phillipsburg, NJ). Both solvents were HPLC grade.

Test Probes

Bovine pancreatic ribonuclease Type 1-A No. R-4875, insulin No. I-5500, serum albumin (BSA) No. A-7511, horse heart cytochrome *c* Type III No. 2506, chicken egg albumin Grade V No. A-5503, and L-tryptophan were purchased from Sigma Chemical Company (St. Louis, MO). Test solutions were prepared in 0.1% TFA (v/v).

Preparation of Stationary Phases

n-Alkylation of silica via silylation was achieved by methods analogous to those previously reported (13,14).

Elemental Analysis

Carbon and hydrogen analyses were performed by C. S. Yeh, Chemistry Department, Purdue University. Accuracy was $\pm$ 0.2%.

Column Packing

Supports were slurry packed (2% w/v) into columns with 2-propanol at 8000 psi by means of a Haskel model 29891 pneumatic

pump, (Haskel Engineering and Supply Co., Burbank CA). All columns were 0.41 x 5.0 cm LiChroma stainless-steel precision tubing (Anspec Co., Inc., Warrenville, IL) with 2.0 µm frits. A precolumn (0.41 x 4.5 cm) was fitted to the slurry vessel and connected to the analytical column to channel silica into the column during the packing process. Packing time was ca. 10 min.

High-Performance Liquid Chromatography

Analyses by HPLC employed a Varian 401 Vista System (Varian Associates, Walnut Creek, CA). The program allowed the microprocessor- controlled data system to tabulate resolution and internal standard recovery measurements directly. The system was fitted with a Valco model 9080 sample injector (Anspec Co., Inc., Warrenville, IL) with a 100 µl injection loop. Protein elution was accomplished by application of a binary gradient in which the primary mobile phase was 0.1% (v/v) TFA in water and the secondary phase was either 0.1% (v/v) TFA in 2-propanol or acetonitrile. All elutions were at a flow rate of 0.7ml/min at ambient temperature. Theoretical plate values were determined by the $NaNO_3$ method (15) employing water at 0.5ml/min as the mobile phase and a sample load of 0.1 µg $NaNO_3$ in 10 µl water. An Altex model 110A pump and model 150 254nm fixed wavelength detector (Altex Scientific Inc., Berkeley, CA) with the Valco value previously described were used for efficiency determinations.

Loading Study Protocol

Ovalbumin was used for all column capacity determinations. The method consisted of 800 µg injections every two minutes until the

breakthrough peaks attained a constant integrated area. The mobile phase was 0.1% (v/v) TFA at 0.7 ml/min and ambient temperature. A standard curve of peak area vs. amount of ovalbumin injected was used to determine column protein capacity. Precision was ± 2%. Columns were regenerated by at least nine blank gradient washes.

RESULTS AND DISCUSSION

A series of n-alkyltrichlorosilanes (C_2, C_4, C_8, C_{12}, C_{18}, C_{22}) were bonded to macroporous silica under identical reaction conditions and packed into columns. The freshly packed columns were washed with three blank gradients prior to the ovalbumin (OVA) loading experiment. The initial column capacity for ovalbumin ranged from 14.45 mg for C_2 to 19.35 mg for C_8 columns (Table 1). The fact that the C_8 chain had a higher capacity than C_{12}, C_{18}, and C_{22} suggested that longer chains folded

TABLE 1

n-Alkyl Chain Length vs. Loading Capacity

ligand	initial OVA load (mg)	second OVA load (mg)	% change in capacity
C_2	14.45	14.42	0
C_4	18.47	19.29	4
C_8	19.35	19.19	-1
C_{12}	17.25	8.84	-49
C_{18}	15.33	11.99	-22
C_{22}	16.98	13.16	-23

over and self-associated as postulated in thermodyamic studies (16-18). Since new columns have a tendency to irreversibly bind some protein upon initial injections (19), a loading correction was made. The ovalbumin was eluted with nine successive TFA/propanol gradients and the loading was repeated. The second set of values should reflect normal column conditioning. It was interesting to note that chain lengths longer than C_8 had a significantly reduced loading capacity. This suggested that longer chains tended to irreversibly bind more protein during initial "conditioning" injections. The decrease in C_{12} protein capacity was very pronounced and could be due to the fact that it had the lowest carbon load of all columns tested (Table 2). Low carbon loading usually indicates a proportionally higher residual surface silanol content, which could be responsible for nonspecific silica-protein adsorptive interactions during the initial loading. Interestingly, the low carbon content did not affect protein retention (Table 2), but may be responsible for observed resolution effects (Table 3). Both C_4 and C_8 chains had capacities of about 19mg on initial and second loadings. The loading capacity of the C_2 column was reproducible but less than C_4 or C_8 columns. After conditioning, the long n-alkyl chains ($\geq C_{12}$) had the least capacity, even lower than the C_2 column. This finding is directly contradictory to what one would expect for small molecules, where increasing the chain length from C_4 to C_{18} roughly doubles column loading capacity (12). Apparently shorter chains have much less irreversible or tenacious retention of ovalbumin. This more

Table 2

Protein Retention vs. n-Alkyl Chain Length

Column	Elemental Analysis		Retention Time[1] (min)					
ligand	%C	%H	tryptophan	ribonuclease	insulin	cytc	BSA	OVA
C_2	3.22	1.24	2.3	16.5 min	17.8 min	20.4 min	23.0 min	26.8 min.
C_4	3.66	1.37	5.0	17.5	18.5	21.7	23.5	28.0
C_8	5.52	1.74	7.2	17.2	17.8	21.6	23.8	27.9
C_{12}	2.99	1.18	2.1	17.0	18.6	21.3	23.9	27.7
C_{18}	4.16	2.34	5.6	16.8	17.5	20.5	23.3	27.2
C_{22}	9.54	2.32	7.1	17.0	18.0	20.8	23.4	27.5

[1]Gradient conditions as in Fig. 1b.

TABLE 3

Protein Resolution vs. N-alkyl Chain Length

Column ligand	Column Efficiency N	Resolution Combination				
		$R_s$rib/insl	$R_s$insl/cytc	$R_s$cytc/BSA	R_sBSA/OVA	ΣR_s
C_2	52.7	1.5	2.7	2.7	4.3	11.2
C_4	35.8	1.1	3.0	2.7	4.9	11.7
C_8	41.4	0.6	3.6	2.2	3.9	10.3
C_{12}	44.7	1.1	1.8	2.2	2.5	7.6
C_{18}	24.6	0.7	2.9	2.6	3.5	9.7
C_{22}	37.1	0.8	2.2	2.6	3.3	8.9

[1]Gradient conditions as in Fig. 1b.

complete desorption has been noted by Nice et al (11) where "ghost peaking" was notably diminished when shorter chains were employed. The cause of irreversibe protein binding is not known. One might suspect partial unfolding of the proteins and tight association of the hydrophobic core with the C_{18} network but not with short alkyl chains.

An interesting observation was that chain length did not affect protein retention time during analytical gradient elutions employing a TFA/2-propanol mobile phase system. Individual retention times for ribonuclease, insulin, cytochrome c, BSA, and ovalbumin were relatively constant for columns ranging from C_2 to C_{22} and in carbon loadings from 2.99 to 9.54%C (Table 2, Fig. 1). The retention times for tryptophan, on the other hand, were consistent with earlier observations for small molecules; retention time increased with %C loading (12). This finding coupled with previous column length studies (13,14) indicated a multisite interaction between protein and alkyl chains, independent of alkyl chain length. The data strongly suggest that proteins only interact with the extreme top portion of alkyl chains under these mobile phase conditions.

Resolution values of adjacent peaks for the five protein test probes showed that no particular column was optimal for each pair of proteins, although a sum total of resolution values indicated that the C_4 column was the most versatile (Table 3). No correlation existed between resolution and column efficiency for the various alkyl chains. A similar conclusion was reached when silica type was

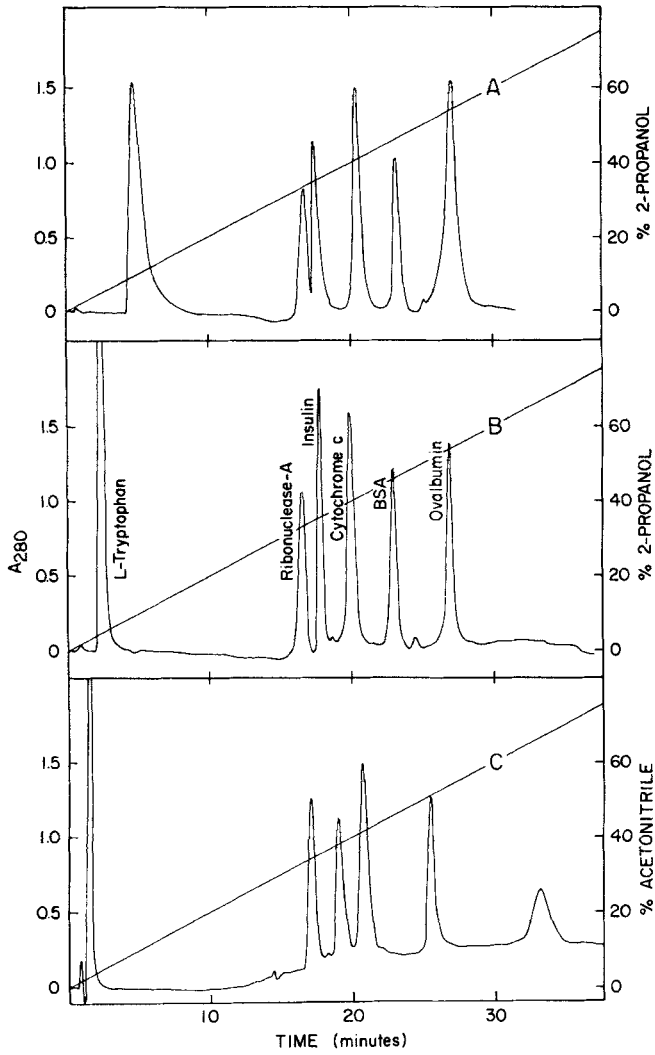

Figure 1 Effects of n-alkyl chain length and mobile phase type on protein retention times. 0.41 x 5cm columns; 0.7 ml/min flow; ambient temperature; 100 µl injection volume; sample sizes in order of elution, 10 µg, 160 µg, 95 µg, 80 µg, 145 µg, and 140 µg. All chromatograms have the same elution order as defined in B. A: C_{18} column, solvent system was 0.1% (v/v) TFA in H_2O into 0.1% (v/v) TFA in 2-propanol. Gradient was 0-80% solvent B in 40 min. B: C_2 column, same elution condition as in A. C: C_2 column, same elution conditions as in A except acetonitrile replaced 2-propanol.

the variable and chain length was held constant at C_8 (13). Acetonitrile offered better selectivity than 2-propanol in some cases (compare Fig. 1B with 1C).

A recent review (20) noted that less than 2% of all reversed-phase separations reported in the literature were done with short alkyl chain supports. This is probably due to early chain length studies which showed better selectivities of chains $\geq C_8$ for a variety of small molecules (21-24) including small peptides (25). Issaq (26) has stated that early studies made no attempt to optimize separation by normalizing the ratio of binary mobile phase constituents to fit relative hydrophocities of the various alkyl chain lengths. When these adjustments were made, Issaq found that short chains could have comparable selectivity (26). Such normalization adjustments were not required in this study because protein retention was found to be independent of chain length. Nevertheless, the utility of short n-alkyl chains in RPLC has thus far not been fully exploited.

In conclusion, protein binding capacity was found to be greatest on C_4 and C_8. Protein retention time for analytical separations was independent of chain length and stationary phase carbon content, while resolution was optimal on the C_4 column. It is evident that short n-alkyl chains can have advantages over C_{18} for protein fractionation.

Note added in proof

The utility of short alkyl chain columns has recently been demonstrated for HPLC of transfer ribonucleic acid molecules (21)

where isoacceptor species have been fractionated in less than an hour.

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[1]Abbreviations used: RPLC, reversed-phase liquid chromatography; TFA, trifluoroacetic acid; OVA, ovalbumin; BSA, bovine serum albumin.

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