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EFFECTS OF CHAIN LENGTH AND CARBON LOAD ON THE PERFORMANCE OF ALKYL-BONDED SILICAS FOR PROTEIN SEPARATIONS

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SUMMARY

The retention and recovery of proteins in reversed-phase liquid chromatography with gradient elution using aqueous–organic mobile phases were found to be similar from large pore (30 nm) silica bonded to maximal extents with either propyl- or octyldimethylsilanes. Proteins were less retained and less completely recovered from silica partially bonded with octyldimethylsilane when used with an acetonitrile-containing mobile phase but not with 1-propanol as modifier. The elution position of proteins under gradient conditions was shown to be independent of flow-rate for fixed gradient slope, but to move later in the gradient with increases in gradient slope. The effects of gradient slope and flow-rate on resolution, separation time, and sensitivity are illustrated.

INTRODUCTION

Gradient elution from alkyl-bonded silica is well established as a powerful analytical and preparative separation method for peptides and small proteins[1,2]. Early in their evolution these general methods had been extended to the separation of larger molecules (*i.e.*, proteins with molecular weights > 10,000 daltons)[3]. Only more recently, however, have studies of the optimization of stationary phases and chromatographic conditions specifically for these larger molecules been reported. The effects of varying pore size[4,5], nature of the bonded phase[5–7], and mobile phase composition[6,8,9] on efficiency, retention, and selectivity have been examined.

Another performance criterion of this separation method applied to proteins which is now recognized to depend sensitively on chromatographic conditions is

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recovery[10]. With non-optimal conditions, incomplete recovery is often evident from the appearance of ghost peaks on repeated gradients. If unsuspected, such behavior could seriously confound the interpretation of biological investigations. An understanding of the dependence of recovery on separation conditions is therefore essential in order that methods which minimize problems of poor recovery can be developed.

In an earlier study of 8–10-nm pore alkyl-bonded silicas differing in length of alkyl chain, but also in base silica and method of preparation, we had found that a short chain (C_3) enabled the separation of a greater range of proteins and afforded best recovery[7]. In the present study undertaken to further define the optimal alkyl-bonded stationary phase for this mode of protein chromatography, we have compared the retention, selectivity, and recovery obtained on a large-pore (30 nm) silica bonded with a C_3 or C_8 alkyl chain. Present evidence indicates that recovery and retention on these maximally bonded C_3 and C_8 stationary phases are similar. Additional results show that the extent of coverage is an important determinant of recovery. In light of these results and the fact that the previously studied packings were not all from the same manufacturer, differences in recoveries from the C_3 and C_8 packings found earlier likely reflect differences in addition to chain length (*e.g.* different silicas, bonding methods, etc.).

It is also known that recovery depends on flow-rate, gradient slope, temperature and sample size[10]. Because varying flow-rate or gradient slope to optimize recovery may require compromises in efficiency or detectability, we have also examined the effects of these variables on the resolution of a test mixture of proteins.

MATERIALS AND METHODS

The chromatographs employed consisted of Beckman Model 100 or 112 pumps, a Model 420 system controller, a Model 210 injection valve, high-pressure dynamic mixer, and a Model 160 absorbance detector operated at 280 nm. Chromatograms were recorded on either an Altex C-RIA recording integrator or a Houston Instruments Superscribe recorder.

All experiments were performed on 7.5 cm × 4.6 mm I.D. stainless-steel columns packed with one of the alkyl-bonded stationary phases listed in Table I. The base silica used was spherical with a mean particle diameter of 10 μm, mean pore size of 30 nm, and a surface area of 91 m^2/g. The extent of alkylsilylation was calculated from carbon analyses corrected for a small amount of carbon in the unbonded silica (Galbraith Labs., Knoxville, TN, U.S.A.), and surface areas determined by a 3-point nitrogen adsorption method (Quantachrome, Syosset, NY, U.S.A.)[11].

Proteins chromatographed included ribonuclease A (RNase A) from bovine pancreas, cytochrome *c* (Cytc) from equine heart, bovine serum albumin (BSA), carbonic anhydrase (CA) from bovine erythrocytes, and hen egg albumin (Oval) (all purchased from Sigma, St. Louis, MO, U.S.A.), and hen egg lysozyme (Lyso) and human transferrin (Trans) purchased from Calbiochem (La Jolla, CA, U.S.A.). Linear aqueous–acetonitrile or 1-propanol (Burdick and Jackson, Muskegon, MI, U.S.A.) gradients with a constant concentration, 0.01 *M*, of trifluoracetic acid (TFA) (Pierce, Rockford, IL, U.S.A.) were used in all cases. All chromatography was performed at 30°C by circulating water from a thermostatted bath through glass column jackets.

TABLE I

STATIONARY PHASES

Chain length	*Trimethylsilyl capped*	*Carbon (%)*	*Coverage (μmoles/m²)*
C_3	+	2.5	4.7
C_3	−	2.5	4.7
C_8	+	3.6	3.4
C_8	+	1.8	1.7
C_8	−	1.8	1.7

Protein recoveries were determined under gradient conditions (1 %/ml) at a flow-rate of 1 ml/min. Recoveries were estimated by comparing areas of eluted peaks with areas obtained when the column was replaced by approximately 10 m of tightly coiled 0.5 mm I.D. PTFE tubing and the same amount (10 to 20 μg) of each protein was injected at 1 ml/min. The profiles of peaks obtained from the 10 m length of PTFE tubing were reasonably symmetrical and of heights similar to peaks eluted from the columns. Protein not recovered from columns was largely removed by a steep gradient (2.5 %/ml) at a high flow-rate (1.5 ml/min) —conditions known to maximize recovery— between each injection of protein[10]. The absence of systematic changes in recoveries from successive injections indicated that the intervening rapid blank gradients effectively eluted all proteins from the columns. In cases of low recovery, ghost chromatograms were produced by the blank gradients. The failure of second blank gradients (occassionally run) to produce significant ghost peaks further confirmed that sample carry-over between injections was not occurring. Corrections for solvent effects on protein absorbance (corrections were 10 % or less) were made by measuring peak areas obtained on the columnless system with various amounts of modifier in the mobile phase. The validity of this method provided it is not applied to excessively tailing or otherwise distorted peaks was established in earlier studies by comparisons with methods based on a tryptophan internal standard and a dye binding protein assay[7,10]. Although the accuracy of the method, estimated to be not worse than ±15 %, is less than more direct methods it is nevertheless adequate for the purposes employed and has the merit of being very convenient. All data shown are averages of 3 or more replicates.

RESULTS AND DISCUSSION

The mobile phase compositions at the column outlet for each protein at the time of elution, calculated accounting for the gradient delay in the mixing chamber and the column dead volume, are listed in Table II and III. Each of these values is an average of 3 or more observations. The repeatability of these data with a single column on the same instrument was better than ±2 %. Repeatability between different columns of the same type was typically ±5 %.

Because of the very sensitive dependence of protein retention on modifier concentration[12], small variations between the gradients produced by different chromatographs, which result from slight differences in relative pump rates or system dead volumes, can cause differences in elution times for the same column on different

TABLE II

% ACETONITRILE AT ELUTION

Column	*RNase A*	*Cytc*	*Lyso*	*Trans*	*BSA*	*CA*	*Oval*
C_3, Maximal coverage, capped	26.9	30.9	34.4	36.5	38.4	42.1	47.8
C_8, Maximal coverage, capped	26.6	31.5	34.8	36.5	37.8	42.1	46.8
C_8, Partial coverage, capped	22.3	27.9	30.8	32.9	36.6	37.7	42.8
C_8, Partial coverage, uncapped	21.9	28.1	30.2	33.4 coeluted		36.7	43.0

instruments on the order of $\pm 5\%$. All of the data shown in Table II and III were obtained using a single instrument.

The data in Tables II and III indicate that the retentions of proteins on maximally bonded and trimethylsilyl (TMS) capped C_3 and C_8 stationary phases are virtually the same. Comparisons of retention times for maximum coverage capped and uncapped C_3 columns obtained on the same chromatograph (a different instrument than used for the data in Tables II and III, data not shown) revealed identical ($\pm 2\%$) retentions for all proteins tested. Apparently the residual silanol population on a maximally bonded C_3 packing is either small or inaccessible to proteins as no effect of TMS capping these maximally bonded stationary phases was observed.

Compared to the maximally bonded packings, the retentivities of the partially bonded C_8 packings, whether capped or not, were uniformly lower with acetonitrile as modifier (Table II). These data are consistent with the concensus that retention of proteins on alkyl-bonded silica is predominatly by hydrophobic interaction[7]. These data also suggest however, that the apparent independence of protein–stationary phase interactions on chain length within the range of 3 to 8 carbons may not extend to the shortest chain, *i.e.*, a TMS stationary phase. Although the data are too few to permit definitive conclusions the difference in protein retention on the maximally bonded C_3 and partially bonded C_8 packings compared to the similarity in their carbon loads in terms of alkyl chain carbons (*i.e.* 4.7 μmoles/m^2 $\times$ 3 carbons and 1.7 μmoles/m^2 $\times$ 8 carbons) does not suggest a correlation of retention with carbon load.

TABLE III

% 1-PROPANOL AT ELUTION

Column	*RNase A*	*Cytc*	*Lyso*	*Trans*	*BSA*	*CA*	*Oval*
C_3, Maximal coverage, capped	15.9	19.9	20.8	22.3	22.7	26.2	30.2
C_8, Maximal coverage, capped	15.9	20.2	21.0	22.3	23.2	26.7	31.2
C_8, Partial coverage, capped	15.4	20.9	21.2	23.3 coeluted		26.3	30.7
C_8, Partial coverage, uncapped	16.8	22.2	22.6	25.4 coeluted		28.4	33.7

With 1-propanol as modifier there were also no detectable differences in protein retention between the maximally bonded C_3 and C_8 stationary phases. Unlike the data with acetonitrile as modifier, however, protein retention on the partially bonded C_8 packings was also very similar to retention on those maximally bonded. As propanol is a stronger reversed-phase solvent than acetonitrile it will distribute to a greater extent into the stationary phase than acetonitrile[13] and will also interact more strongly than acetonitrile with accessible silanol sites which exist in substantial density on the uncapped partially bonded packing. Thus, differences between the various stationary phases may be diminished when solvated by a propanol containing mobile phase relative to a mobile phase containing acetonitrile. Taken together, the data in Tables II and III indicate that the retention of proteins, perhaps because of their large size, is less dependent on the characteristics of bonded alkyl surface than small solutes[14].

It is important to note that the resolution of transferrin and BSA was lost on the partially bonded uncapped packings with both acetonitrile and propanol, and also on the partially bonded, capped packing with propanol. The apparent efficiencies of the C_3 and C_8 maximally bonded packings were similar and both produced peaks with widths noticeably narrower than the partially bonded packings. The reproducibility of the uncapped columns was poorer than the other stationary phase, especially with regard to recovery. Not surprisingly, the number of injections that could be made on the partially covered uncapped columns before performance and retention behavior changed markedly (decreases $\geq 10\%$) was small (< 50 injections). By contrast the performance of maximally covered columns was essentially unchanged after over 200 injections.

The recoveries of ovalbumin from the various stationary phases with an acetonitrile–TFA gradient are listed in Table IV. Recoveries from the 3 maximally bonded stationary phases are, within the uncertainty of the method used, the same. The somewhat lower recovery obtained on the partially bonded, but capped C_8 packing is a consistent observation and therefore probably real, however limitations on the accuracy of the method used preclude more definitive conclusions. Recovery from the uncapped packing was more variable, but always lower than 40%. The less hydrophobic proteins, RNase A and BSA were recovered in acceptable yields (90–100%) from all the stationary phases tested.

TABLE IV

PROTEIN RECOVERY

Column	*Recovery (%)*
C_3, Maximal coverage, capped	77
C_3, Maximal coverage, uncapped	76
C_8, Maximal coverage, capped	78
C_8, Partial coverage, capped	69
C_8, Partial coverage, uncapped	23

The similarity of the recoveries obtained from the maximally bonded C_3 and C_8 packings compared with results of an earlier study in which substantially poorer recoveries were observed on packings with chain lengths longer than C_3 (ref. 7) requires some comment. As the earlier comparisons were between available bonded stationary phases from various sources, the differences in recovery from C_3 and C_8 packings observed in that study likely reflect differences in the physical and chemical characteristics of the silicas involved and/or differences in the methods used to prepare the bonded stationary phases rather than an effect of chain length. There are two other differences between the two studies which could also account for the different dependencies of recovery on chain length. The mobile phase used in the first study, 0.155 *M* sodium chloride solution acidified to pH 2.1 with hydrochloric acid, is less effective for the recovery of hydrophobic proteins than the dilute TFA used here[10]. Also, the pore sizes of the silicas used in the two studies were different —8–10 nm compared to 30 nm. The present data do not exclude the possibility that an effect of chain length on protein recovery develops only on small pore packings. There remain in the earlier study comparisons of recoveries from columns which were equivalent in all respects except chain length and which indicate significantly poorer recoveries from octadecyl than from shorter chain packings. Specifically, recovery of ovalbumin was greater on LiChrosorb RP-8 than on LiChrosorb RP-18, and greater on Ultrasphere C_3 than on Ultrasphere ODS (C_{18}). Thus recovery of hydrophobic proteins from alkyl bonded silica columns appears to depend on several characteristics of the stationary phase.

It has also been shown that the recovery of problematic proteins (*e.g.* ovalbumin) from these types of columns depends not only on the particular packing and eluate used, but also on flow-rate and gradient slope[10]. As most protein separations on such columns are performed at very high reduced velocities where recovery is good[10] but efficiency may be compromised[15], it seems probable that both optimal recovery and resolution will not always be achieveable. While the dependence of resolution and efficiency on flow-rate and gradient has been studied by others[15,16], it seemed useful to examine the effects of varying these conditions on the large-pore packings which had been characterized with respect to recovery.

Snyder[17] has provided a fundamental definition of gradient steepness, φ'', which for a linear gradient can be expressed as

$$\varphi'' = (t_0/t_G)\ (\text{final \% modifier} - \text{initial \% modifier}) \tag{1a}$$

$$\varphi'' = t_0\ (\Delta\ \%\ \text{modifier/min}) \tag{1b}$$

where t_0 is the column dead time and t_G is the gradient time (min). The appearance of t_0 in these expressions makes it clear that gradient steepness is not completely specified by the change in volume % modifier per unit time. Because t_0 depends on flow-rate, column dimensions and porosity, gradient slope is also a function of these variables. For the purposes of this study, since column dimensions were not varied and column porosities could reasonably be assumed constant, it is more convenient to rewrite eqn. 1b as

$$\varphi'' = v_0\ (\Delta\ \%\ \text{modifier/ml}) \tag{2}$$

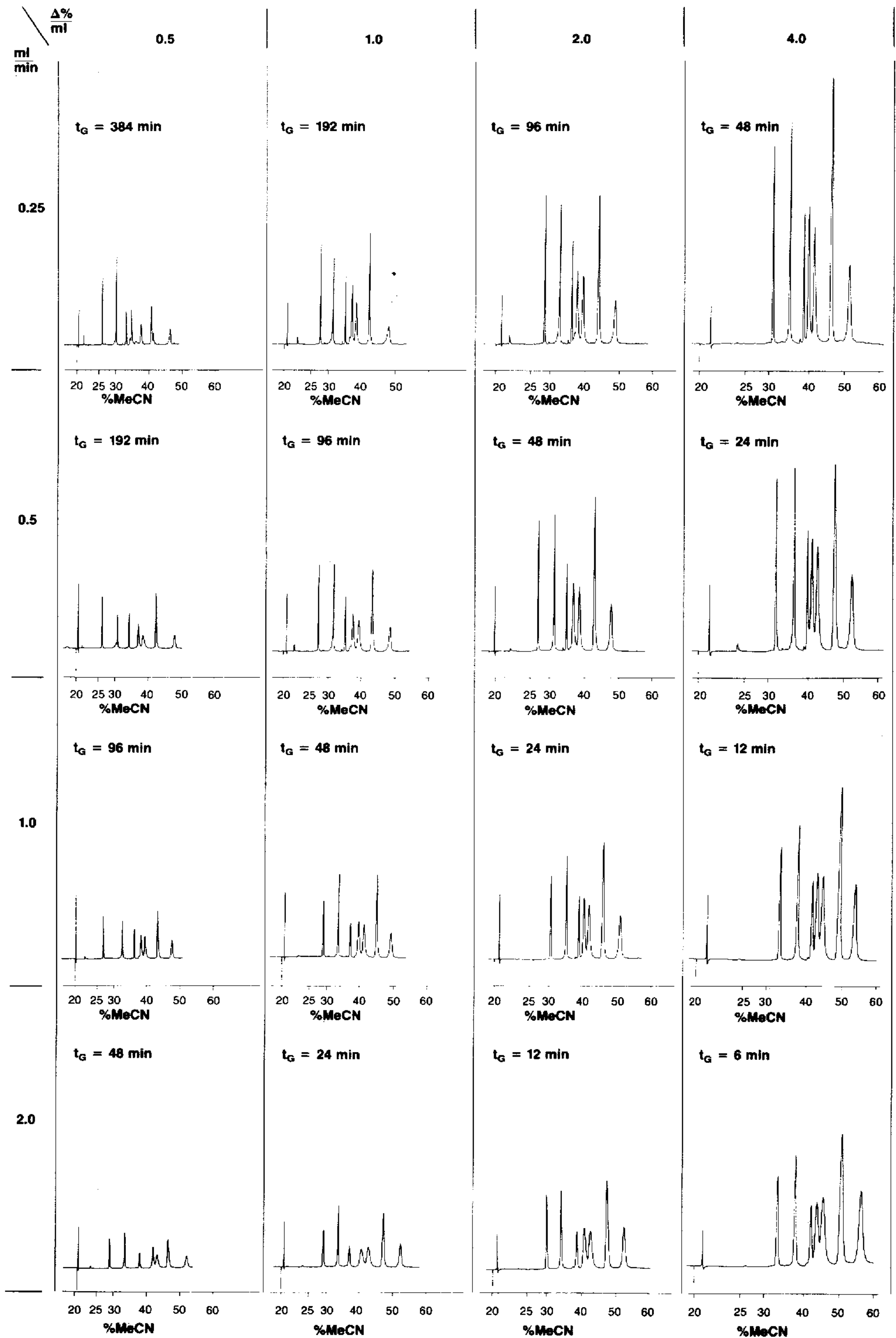

Fig. 1. Effects of flow-rate and gradient slope in reversed-phase protein separations. The peaks, in order of elution, are RNAse A, Cytc, lysozyme, transferrin, BSA, carbonic anhydrase, and ovalbumin (each ca. 5 μg). Aliquots of the same sample were used in all cases. The linear gradient was from 20% to 68% acetonitrile (MeCN) in 0.01 *M* TFA over the times indicated. Recorder speed was normalized to gradient time. Detection was by absorbance at 280 nm, 0.1 a.u.f.s. in all cases.

where v_0 is the column dead volume in milliliters. The interpretation of these definitions is that gradient steepness is best expressed as the change in % modifier per passage of mobile phase volume equal to v_0. Because v_0, unlike t_0, is independent of flow-rate, gradients with equal changes in % modifier per milliliter will, for columns of the same dimensions, be equivalent. Accordingly the effects of varying flow-rate and gradient slope are illustrated in Fig. 1 where the separations in each row were carried out at fixed flow-rate and varying gradient, and those in each column at fixed gradient slope but varying flow. It is noteworthy that the same separation, albeit with significant differences in resolution and detectability, could be achieved over a 64-fold range of gradient times.

The abscissa in each panel of Fig. 1 is expressed in % acetonitrile calculated accounting for the mixing chamber volume. In agreement with theory[17], for a given gradient slope each protein eluted at approximately the same mobile phase composition regardless of flow-rate, but progressively later in the gradient (*i.e.* at greater modifier concentration) as gradient steepness was increased.

The shortest separation time is achieved using both a fast flow-rate and a steep gradient. The chromatogram in the lower right corner illustrates that considerable resolution can be achieved much faster than is commonly practiced. Greatest resolution (upper left corner) is obtained by using a slow flow-rate and a shallow gradient — conditions which, however, prolong separation time and may compromise recovery. A very evident effect among the 16 chromatograms in Fig. 1 is the variation in peak height. Peak height increases both with decreasing flow-rates where column efficiency is greater, and also with increasing gradient steepness where the capacity factor at elution is smaller and peak compression is greater. Thus the increases in peak height which occur with increasing gradient steepness (left to right in each row of Fig. 1) are in qualitative agreement with practical theory[18]. Further studies which would permit a more quantitative comparison with theory would clearly be useful.

Clearly, optimal conditions cannot be specified without considering a specific separation problem. However, it seems likely that commonly used conditions, *e.g.* 1 ml/min and 1 %/min for a 15–25 cm column, could sometimes be altered to significantly shorten separation time if resolution permitted, to increase resolution if a longer separation time can be tolerated, or to increase peak heights (detectability) without changing analysis time by decreasing flow-rate and increasing gradient slope. A more detailed discussion of these considerations can be found in a report by Karger[18].

Finally, returning to the issue of recovery, although no quantitative determinations were made in this particular set of separations (Fig. 1), a comparison of peak heights of the last eluting peaks relative to the first few in each chromatogram suggests that ovalbumin, the last peak, was recovered more completely at higher flow-rates and steeper gradients than otherwise in agreement with previous results.

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