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COMPARISON OF SHORT AND ULTRASHORT-CHAIN ALKYLSILANE-BONDED SILICAS FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS BY HYDROPHOBIC INTERACTION METHODS

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SUMMARY

The optimization of the reversed-phase high-performance liquid chromatography of proteins has been examined using a series of maximum-coverage alkylsilane-bonded silica packings with different carbon chain-lengths (C_1–C_{18}). The greatest range of individual compounds that could be successfully chromatographed was obtained with $< C_6$ chain-length packings. Optimum recoveries and efficiencies were noted with a C_3 material (Ultrasphere SAC). No loss of performance or changes in selectivity were observed during prolonged use (> 200 h) of these columns under acid (pH 2.1) conditions. This system has been used to separate prolactin and growth hormone (22 kD) from biological samples by employing a trace-enrichment step on 10-μm tap-packed short alkyl-chain columns prior to gradient-elution chromatography on a 5-μm particle-size (8 nm pore-size) Ultrasphere SAC column.

INTRODUCTION

One of the most powerful applications of high-performance liquid chromatography (HPLC) to emerge in recent years in respect of biological molecules has been the separation of peptides using alkylsilane-bonded and related reversed-phase (RP) type materials. Although these techniques have, for the most part, been used for the separation of relatively small molecules, larger polypeptides[1,2] and indeed small proteins[3,4] can be successfully chromatographed. As with the smaller polypeptides it appears that protein separations are dictated primarily by the interaction of their more accessible hydrophobic constituents or domains with the stationary phase.

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TABLE I

SOURCES AND DETAILS OF POLYPEPTIDE AND PROTEIN STANDARDS USED

Compound	*Supplier*	*Mol.wt. (kD)*	*Abbreviation used*
Calcitonin (synthetic human)	Ciba (Basle, Switzerland)	3.4	hCT
Epidermal growth factor (mouse)	Collaborative Research (Waltham, MA, U.S.A.)	6.0	mEGF
Parathyroid hormone (bovine)	Dr. J. Zanelli (National Institute for Biological Standards and Control (NIBSC), Holly Hill, Hampstead, Great Britain)	9.5	bPTH
Cytochrome *c* (horse heart type III)	Sigma (Poole, Great Britain)	11.7	Cyt *c*
Ribonuclease A (bovine pancreas type IIIA)	Sigma	14.2	RNase
α-Lactalbumin (bovine)	Sigma	14.2	b α-Lact
α-Lactalbumin (rat)	Dr. K. E. Ebner (University of Kansas, Kansas City, U.S.A.)	14.2	r α-Lact
Lysozyme (hen eggwhite 3× recryst, A grade)	Calbiochem-Behring (Bishops Stortford, Great Britain)	14.3	Lys
^{14}C-Methylated lysozyme*	Amersham International (Amersham, Great Britain)	14.3	
α-Hemoglobin (bovine)	Dr. J. Zanelli	15.1	αHb
Myoglobin (whale muscle)	Calbiochem-Behring	17.2	Myo
β-Lactoglobulin A (bovine milk)	Sigma	18.3	Lactog A
β-Lactoglobulin B (bovine milk)	Sigma	18.3	Lactog B
Growth hormone (bovine GH-B-18)	National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD), Pituitary Hormone Distribution Program (Bethesda, MD, U.S.A.)	22.0	bGH
Growth hormone (rat GH-B-6)	NIAMDD	22.0	rGH
Human placental lactogen	ICN Pharmaceuticals (Cleveland, OH, U.S.A.)	22.1	hPL
Prolactin (ovine P-S-13)	NIAMDD	22.6	oPRL
Prolactin (rat PRL-B-2)	NIAMDD	22.6	rPRL
Elastase (porcine pancreas type III)	Sigma	25.9	Elas
Carbonic anhydrase (bovine erythrocytes)	Sigma	29.0	CA
^{14}C-Methylated carbonic anhydrase*	Amersham International	30.0	
Ovalbumin (hen eggwhite, fraction V)	Sigma	45.0	Ovalb
Human serum albumin (fatty acid free)	Sigma	66.5	HSA
Bovine serum albumin (fraction V)	Sigma	68.0	BSA
^{14}C-Methylated bovine serum albumin*	Amersham International	69.0	

* Prepared by reductive alkylation with [^{14}C]formaldehyde and sodium cyanoborohydride at pH 7.0 (ref. 10).

Experience in the optimization of such RP-systems specifically for protein chromatography is, however, still limited[5]. The majority of comparable studies with peptides and polypeptides have utilized long-chain packings of the C_{18} (octadecylsilane) type. Some large polypeptides, such as, for example, bovine[2] and human[6] parathyroid hormone (9.5 kD) can be successfully chromatographed with such supports, as can some even larger materials (<69 kD)[3,4]; it is, however, our experience that many small proteins well below the nominal exclusion limit of these mesoporous (6–10 nm) silica-based packings either cannot be eluted[3], or give poor recoveries with these systems.

The use of C_8 (octylsilane) RP-packings and solvents such as propanol have both been shown to be advantageous for the separation of this type of compound[7]. The use of other shorter chain alkylsilane-bonded materials for protein chromatography by these "hydrophobic interaction" RP-HPLC methods has not, however, been systematically investigated. We report here such a study, and show that certain other short-chain alkylsilane-bonded silica packings afford additional advantages for protein HPLC. Not only can a larger number of individual compounds be chromatographed, but overall recoveries of proteins are also enhanced.

MATERIALS AND METHODS

Sources and details of individual protein standards used in this study are given in Table I. An explant culture of a pituitary gland from a lactating rat was used to generate a natural mixture of protein hormones. Nutrient medium from the culture was acidified (pH 2.1) and pumped through an 85 × 5 mm I.D. column containing tap-packed LiChrosorb RP-2 (as Table II except 10-μm particle size) to trace-enrich the hormones therein. Adsorbed proteins were eluted using a step gradient of acetonitrile in pH 2.1 0.155 *M* NaCl solution, as described in previous studies of high molecular weight calcitonin-like materials[8], and the organic modifier removed by evaporation under nitrogen prior to analytical chromatography of the fraction containing prolactin and growth hormone.

Analytical HPLC of these extracts and of protein standards was carried out with a series of 150 × 5 mm I.D. stainless-steel columns packed with each of the supports listed in Table II. Operating conditions were controlled using either a Spectra-Physics SP 8000, or an Altex Model 324-40 chromatograph. Dead volumes between the gradient former and column heads were different in these two instruments (low-pressure and high-pressure mixing, respectively), resulting in slight differences in absolute retention times of the proteins using the two systems, under gradient-elution conditions.

Separations were performed at 45°C and a constant flow of 1 ml/min, using continuous linear gradient elution between an aqueous primary solvent of 0.155 *M* NaCl solution adjusted to pH 2.1 with HCl, and a secondary solvent of either acetonitrile, or propan-2-ol (Rathburn Chemicals, Walkerburn, Great Britain). Eluted proteins were detected by UV-absorbance at 215 nm (acetonitrile gradients) or 280 nm (propan-2-ol gradients) (Schoeffel FS 770) and endogenous tryptophan fluorescence, 225 nm excitation, 340 nm emission filter (acetonitrile) or 280/370 nm (propan-2-ol) (Schoeffel FS 970). Radioactive ^{14}C-proteins were detected on-line using a Packard 7500 HPLC monitor. Confirmation of the identity of eluted proteins was obtained

TABLE II

DETAILS OF RP-PACKINGS AND COLUMNS

Packing	*Carbon chain-length*	*Particle size (μm)*	*Pore diameter (nm)*	*Pore vol. (ml/g)*	*Specific surface area (m^2/g)*	*Shape**	*Length (cm)*
Ultrasphere SAS	1	4–6	8	0.57	180	S	15
LiChrosorb RP-2	1	5–7	ND***	ND	500	I	15
Ultrasphere SAC	3	4–6	8	0.57	180	S	15
Spherisorb S5C6	6	3–7	8	0.57	200	S	15
LiChrosorb RP-8	8	4–7	ND	ND	>250	I	15
LiChrosorb RP-18	18	4.3–7.3	ND	ND	>100	I	15
Ultrasphere ODS	18	4–6	8	0.57	180	S	15
Hypersil ODS	18	5–7	10	0.7	200	S	15

* S = spherical, I = irregular.

** PP = packed by manufacturer, LP = packed in laboratory from methanol slurry.

*** ND = not divulged by manufacturer but probably 6–10 nm.

both by polyacrylamide gel electrophoresis of HPLC eluate fractions after evaporation of the organic modifier, and by determination of their retention times on a TSK SW 3000 size-exclusion column (600 × 7.5 mm I.D.) with pH 7.0 phosphate buffer as eluent.

Recoveries of proteins obtained with the different columns tested (Table II) were estimated by one of three methods, depending on the types and quantities of the proteins involved. With standards available in large quantities the protein content of 1-ml eluate fractions was measured directly using a spectrophotometric Coomassie blue metachromatic method (Bio-Rad) with a detection limit, under the conditions used, of >20-μg fraction. The recovery of ^{14}C-methylated proteins was calculated directly from the radioactivity in peak fractions in eluate aliquots measured by liquid scintillation counting. For standards available in more limited amounts, or for which radioactive analogues were not available, an indirect method of estimating recovery was adopted. This was based on the relative areas of the protein peaks on the initial chromatogram, and of the corresponding "ghost" peaks, if any, seen on subsequent "blank" gradient-elution runs. These peaks of incompletely eluted protein diminished in size in sequential blank chromatograms and a linear regression analysis enabled the total amount of protein, and thus the recovery obtained from the initial chromatogram to be calculated. Good agreement (±5%) was obtained between recoveries calculated in this manner and measured directly using the Coomassie blue protein assay.

Recovery of biological activity was monitored in the case of lysozyme using a turbidimetric method[8] and for elastase by the Congo red-elastin assay[9].

RESULTS

Our initial series of experiments was aimed at establishing (1) which individual proteins could be chromatographed on the short and ultra-short alkylsilane-bonded

silica packings listed in Table II, and (2) whether practically useful selective effects could be obtained with individual packings of this type in respect of closely eluted proteins. For the purposes of this study, the results of which are given in Table III, acetonitrile was used as the organic modifier. In most cases between 5–20 μg of each protein was injected. Although propanol affords significant advantages for protein

TABLE III

RETENTION TIMES (min) OF POLYPEPTIDE AND PROTEIN STANDARDS RELATIVE TO LYSOZYME

Short and ultra-short alkylsilane-bonded RP-packings were used under gradient-elution conditions (Spectra Physics SP8000 chromatograph, gradient profile as illustrated in Fig. 1) with acetonitrile as organic modifier and pH 2.1 0–0.155 *M* NaCl as primary solvent. See Table I for abbreviations used. NT = not tested; NR = not recovered using CH_3CN as organic modifier.

Standards	C_1*	C_3**	C_6***	C_8 §
RNase	−10.2	−10.7	−11.3	−12.0
mEGF	NT	− 7.6	− 8.0	NT
hCT	− 4.1	− 4.4	− 4.1	− 5.0
Cyt *c*	− 2.5	− 3.1	− 2.3	− 3.8
bPTH	− 2.4	− 2.3	− 2.1	− 2.6
Lysozyme	0	0	0	0
BSA	3.2	3.3	5.8	7.2
HSA	3.8	3.5	NT	NT
b α-Lact	4.6	7.4	6.6	7.2
r α-Lact	4.9	NT	7.9	NT
Myoglobin	7.1	9.0	9.5	8.9
α Hb	8.2	9.2	9.6	11.3
CA	9.8	9.5	9.6	10.0
Lactog A	10.1	10.0	NT	8.8
Lactog B	10.1	10.0	NT	8.8
Elastase	11.1	10.0	11.7	NR
oPRL	13.1	16.7	16.6	16.6
hPL	14.3	20.2	17.6	18.5
rPRL	17.3	25.0	20.0	29.1
bGH	17.3	29.0	NT	39.4
Ovalbumin	NR	NR	NR	NR

* LiChrosorb RP-2.
** Ultrasphere SAC (experimental).
*** Spherisorb S5C6.
§ LiChrosorb RP-8.

HPLC[7] its UV-absorbance limits the use of low-wavelength detection; consequently a number of high-purity proteins used in this study, and available in only limited quantities could not have been directly detected.

It is evident from Table III that a wide variety of proteins which cannot be chromatographed on C_{18} packings can be successfully recovered from the short-chain materials. Furthermore, while most of the compounds tested could be eluted from.

albeit with varying recoveries, all short and ultrashort-chain packings, at least one protein tested (elastase) could only be recovered from C_6 or shorter-chain materials with acetonitrile as the organic modifier. The identity of the eluted peak was in this instance confirmed by both physical and biological methods (see Materials and methods). Thus, full biological activity was obtained from the protein eluted from the ultra-short chain packings after removal of the organic modifier from the eluent by evaporation.

Comparison of the retention times of these protein standards with that of lysozyme (Table III), under constant conditions of gradient elution with acetonitrile in acid (pH 2.1) saline, showed that while most increased with increasing chain-length (and carbon loading) of the packing, no reversals in retention order indicative of marked changes in selectivity were apparent. Thus by appropriate modification of the slope of the linear gradient profile (see Fig. 1) a virtually identical elution profile could be obtained with all the RP-supports tested. The separation of a series of such standards on a C_3 packing (Ultrasphere SAC) is illustrated in Fig. 1. Fig. 2 shows the UV-absorbtion profile obtained with this column when a trace-enriched extract (see Materials and methods) of tissue culture medium from a lactating rat pituitary gland was chromatographed. The resolution of growth hormone and prolactin (both 22 kD) from this sample and their separation from other proteins was thus readily obtained by the use of RP-HPLC methods.

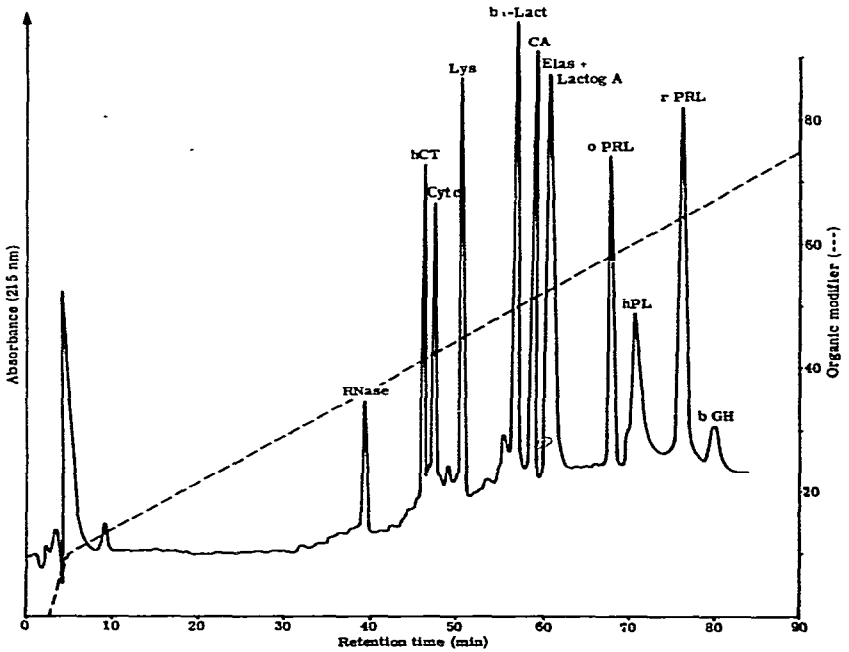

Fig. 1. Separation of protein standards (see Table I for abbreviations) on 150 × 5 mm I.D. Ultrasphere SAC column. Compounds were eluted with a linear gradient of acetonitrile (dotted line) in pH 2.1 0.155 *M* NaCl solution at 45°C and a flow-rate of 1 ml/min (Spectra-Physics SP 8000). The identity of all protein peaks was confirmed by SDS-PAGE.

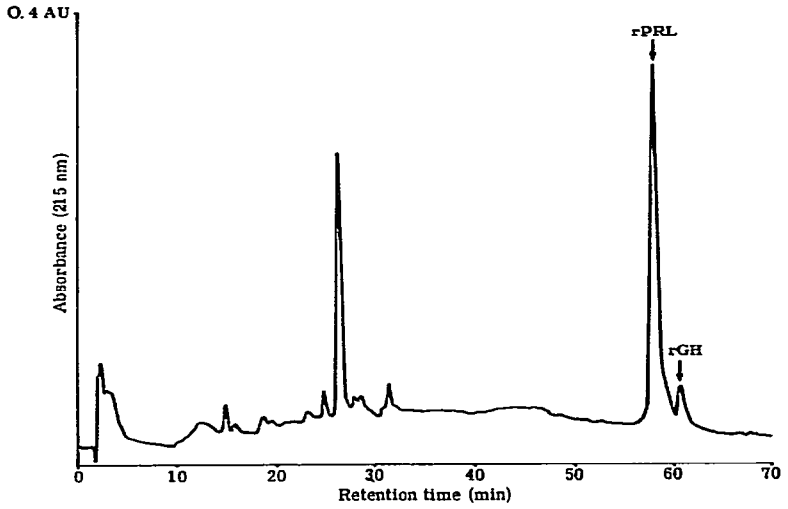

Fig. 2. Separation of hormones secreted by rat pituitary explant culture by RP-HPLC. Conditions of chromatography as Fig. 1, except using an Altex Model 324-40 chromatograph. Note resolution of prolactin (rPRL) and growth hormone (rGH) in this trace-enriched culture medium extract.

No significant loss of efficiency was noted with these packings when the elution volumes of a variety of proteins were compared. The only exception was the ultrashort chain materials (LiChrosorb RP-2, Hypersil SAS) on which the total elution volumes of typical proteins were increased by $<40\%$ compared with the other short-chain materials (C_3–C_8). Preliminary experiments have noted further improvements in efficiency when large pore-size (30 nm) supports of the latter type were used.

The most notable advantage of the short-chain alkylsilane packings apparent from the present study was the improved recovery of many of the proteins tested. This was evident from the size of the "ghost" peaks seen with many compounds on the longer-chain materials, including C_8 packings, and their diminution or complete absence under identical conditions of gradient elution using the shorter chain (*i.e.* C_3) columns. This improvement in recovery is shown in quantitative form in respect of ovalbumin in Table IV. Although this 45 kD protein could not be eluted from any RP-packing tested when acetonitrile was used as the secondary solvent, it could be recovered, in varying yields, using propanol, a solvent which has been shown to have superior properties for protein RP-HPLC by other workers[7]. Recoveries on C_8 packings were, however, low (22%), while with C_{18} columns they were negligible (8%). Optimal recoveries of this protein (78%) were obtained when a C_3 packing (Ultrasphere SAC) was used. With even shorter alkylsilane-bonded phases recoveries were, however, somewhat reduced compared with the C_3 material. Optimal recoveries with the C_3 packing were also seen with a variety of other standards tested, and this stationary phase offered significant advantages in this respect over both C_8 and, to a lesser extent, C_6-type packings.

As the recovery of individual proteins can pose problems with these RP-HPLC methods, the use of an internal standard in the form of a radiolabelled protein might seem of value when processing biological extracts. Examination of the elution profiles

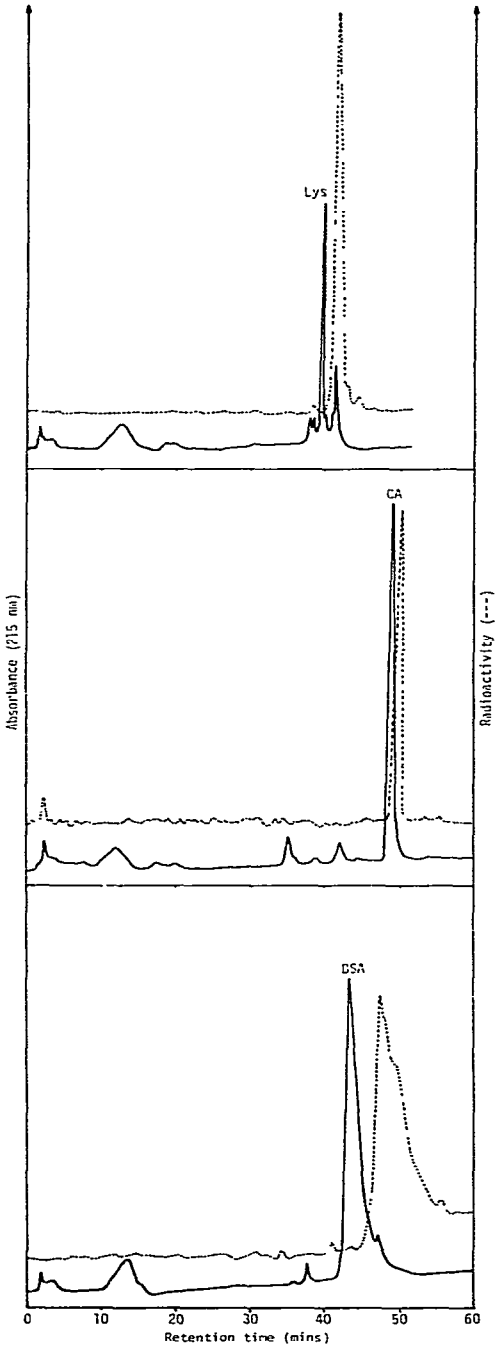

Fig. 3. RP-HPLC of ^{14}C-methylated proteins and their non-radioactive cogeners. Simultaneous UV-absorbtion and radioactivity profiles were determined with conditions of chromatography as in Fig. 1, except for the use of an Altex Model 324-40 chromatograph. Note separation of the radiolabelled proteins from their non-radioactive precursors.

TABLE IV

RECOVERY (%) OF PROTEIN STANDARDS FROM RP-PACKINGS

BSA (50 μg) and ovalbumin (150 μg) were separated on 150 × 5 mm columns using a primary solvent of 0.155 *M* NaCl (pH 2.1) with propan-2-ol as organic modifier and a gradient of the form illustrated in Fig. 1. Recoveries were calculated from the respective peak heights on these chromatograms and those of ghost peaks due to incompletely eluted materials on subsequent blank runs.

Packing	*BSA (65 kD)*	*Ovalbumin (45 kD)*
LiChrosorb RP-2	100	35
Ultrasphere SAC	100	78
Spherisorb S5C6	100	57
LiChrosorb RP-8	100	22
LiChrosorb RP-18	100	8

of ^{14}C-methylated proteins tested (lysozyme, carbonic anhydrase and bovine serum albumin) revealed, however, that all were eluted at a slightly different place on the gradient from their respective unlabelled parent compounds (Fig. 3). Thus, their chromatographic properties differ from those of their parent molecules. They do not, therefore, constitute entirely appropriate internal standards for RP-HPLC when prepared by reductive alkylation[10]. These separations, do, nevertheless, afford another example of the power of these "hydrophobic interaction" methods using optimized RP-HPLC supports for resolving closely related proteins.

DISCUSSION

Although it is now some time since it was first reported[11,12] that proteins could be chromatographed on RP-HPLC systems, it is only recently that attempts have been made to optimize these methods. The results presented here illustrate the wide range of small proteins which can be successfully separated with the use of optimized carbon chain-lengths of the alkylsilane-bonded silica packings and organic modifiers such as propanol[7] which favour the continued solubility of such molecules at relatively high concentrations of secondary solvent necessary to elute proteins under gradient elution conditions. This is, however, unlikely to be a universal method for protein HPLC. Thus, many proteins will still probably be too hydrophobic or too insoluble in the eluting solvents to be successfully chromatographed. Nevertheless, such techniques provide a very useful adjunct to the size-exclusion and ion-exchange HPLC (and "medium" performance) methods recently developed for proteins[5].

The RP-HPLC procedures provide in certain cases a very selective method of both trace-enriching such molecules and separating them from cogeners of similar size and charge. A comparison of the elution order of the proteins we have tested with their net hydrophobicity (Table V) shows that as with polypeptides[3] a general correlation exists, but that numerous individual anomalies occur. These are probably due to conformational differences including the clustering of hydrophobic aminoacid residues as hydrophobic domains[13]; net hydrophobicity does, nevertheless, afford a general guide as to the probability of an individual protein being successfully chromatographed.

TABLE V

THE CORRELATION BETWEEN HYDROPHOBICITY AND ELUTION ORDER OF PROTEINS ON ULTRASPHERE SAC (C_3) UNDER GRADIENT ELUTION CONDITIONS

Protein	*MW (kD)*	*Mole % hydrophobicity**	*Elution order*
Ribonuclease A	13.7	21.8	1
Cytochrome *c*	11.7	25.0	2
Lysozyme	14.3	26.4	3
Human serum albumin	66.5	28.37	4
α-Haemoglobin	15.1	31.20	8
Myoglobin	17.2	31.37	8
Carbonic anhydrase	29.0	31.49	7
Bovine serum albumin	69.0	32.0	5
Elastase$^+$**	25.9	32.5	10
α-Lactalbumin (bovine)**	14.2	32.5	6
Prolactin (ovine)**	22.6	32.8	12
Human placental lactogen**	22.1	33.7	13
Growth hormone (bovine)**	22.0	34.0	14
β-Lactoglobulin**	18.3	34.6	10

* Mole % hydrophobicity is calculated with respect to the following amino acids: Trp, Phe, Leu, Ile, Tyr, Val, Met.

** None of these proteins can be chromatographed on C_{18} packings, using acetonitrile as the organic modifier with pH 2.1 0.155 *M* NaCl as the primary solvent. Elastase$^+$ was not recovered from either C_{18} or C_8 packings tested, under these conditions.

The practical use of these methods is evident from the separations of prolactin and growth hormone that we have obtained (Fig. 2). Preparative separation of these hormones has traditionally posed several problems, not the least of which is the difficulty of obtaining high yields of pure pyrogen-free materials when using time-consuming conventional methods of size-exclusion and ion-exchange chromatography[14]. Hydrophobic HPLC methods would appear to offer significant advantages as they have in the case of parathyroid hormone[2,6].

Carbon chain-length (and carbon loading) are not, of course, the only factors of importance in optimizing RP-HPLC methods for protein chromatography. While all of the packings that we have used here have been capped (where appropriate) or reacted to minimal levels of residual accessible silanol groups[15,16], the possibility of some degree of "mixed mode" chromatography contributing to the differences we have noted between the different packings cannot be entirely excluded. Thus recent studies[17] have shown that low carbon loading, low coverage octadecylsilane packings provide some selective effects with peptide and protein chromatography, although reduced efficiencies were noted unless residual silanol groups were suppressed. However, despite the prolonged exposure of our columns to unbuffered acid conditions, all have afforded useful column lives of >200 h, with highly reproducible chromatography and no changes of selectivity. We believe, therefore, that residual silanols, if any, are probably not responsible for the differences in performance that we have observed.

The use of mobile-phase additives in protein RP-HPLC is complicated by problems in removing them from the eluted compounds after chromatography. Ion-pairing reagents such as the higher perfluoroalkanoic acids[18,19] increase peptide retention; this may afford useful selective effects with smaller compounds but will militate against the successful elution of the more hydrophobic proteins. The use of more polar bonded phases, *e.g.* cyanoalkyl or phenylalkyl-type materials, may assist in the elution of proteins[20] but efficiencies are significantly reduced with at least some of these packings[4]. Another means of reducing the strength of the binding between proteins and alkylsilane phase and thus favouring elution of more hydrophobic molecules is by reducing the temperature, a consequence of the entropic properties of hydrophobic bonds and preliminary studies with sub-ambient temperatures have been encouraging in this respect.

Low flow-rates ($\approx$ 0.3 ml/min) have been reported to enhance the resolution of proteins, increasing efficiency by compensating for the lower diffusion rates of the larger compounds[21]; preliminary results indicate, however, that similar improvements in efficiency may be obtained with larger pore-size silicas (30 nm) without loss of analysis time.

Large pore-size packings also, of course, enable larger proteins to potentially interact with the bulk of the stationary phase. Several recent reports[7,20,22,23] have demonstrated that RP-chromatography of proteins such as collagens ($<$300 kD) are possible with such materials. It remains to be seen, however, how many other large molecules, or mixtures thereof, can be chromatographed; results to date[20] indicate that parameters of the eluting solvent such as pH, may have to be tailored specifically to suit individual proteins. On the basis of the results obtained here ultra-short (C_3) alkylsilane-phases on large pore-size silica may prove suited to such methods.

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