Journal of Chromatography, 548 (1991) 179–193
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2265

Comparison of silica-based cyanopropyl and octyl reversed-phase packings for the separation of peptides and proteins

NIAN E. ZHOU and COLIN T. MANT

Department of Biochemistry and the Medical Research Council of Canada Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta T6G 2H7 (Canada)

J. JACK KIRKLAND

Central Research and Development, E.I. du Pont de Nemours and Company, Experimental Station, B-228, P.O. Box 80228, Wilmington, DE 19880-0228 (USA)

and

ROBERT S. HODGES*

Department of Biochemistry and the Medical Research Council of Canada Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta T6G 2H7 (Canada)

ABSTRACT

The performance of a silica-based C_8 packing was compared with that of a less hydrophobic, silica-based cyanopropyl (CN) packing during their application to reversed-phase high-performance liquid chromatography (linear trifluoroacetic acid–water to trifluoroacetic acid–acetonitrile gradients) of peptides and proteins. It was found that: (1) the CN column showed excellent selectivity for peptides which varied widely in hydrophobicity and peptide chain length; (2) peptides which could not be resolved easily on the C_8 column were widely separated on the CN column; (3) certain mixtures of peptides and small organic molecules which could not be resolved on the C_8 column were completely separated on the CN column; (4) impurites arising from solid-phase peptide synthesis were resolved by a wide margin on the CN column, unlike on the C_8 column, where these compounds were eluted very close to the peptide product of interest; and (5) specific protein mixtures exhibited superior resolution and peak shape on the CN column compared with the C_8 column. The results clearly demonstrate the effectiveness of employing stationary phases of different selectivities (as opposed to the more common optimization protocol of manipulating the mobile phase) for specific peptide and protein applications, an approach underestimated in the past.

INTRODUCTION

The excellent resolving power of the reversed-phase (RP) mode has resulted in its becoming the predominant high-performance liquid chromatographic (HPLC) technique for peptide separations [1,2]. In addition, many protein separations are also effected through this HPLC mode [3,4]. Optimization of peptide and protein separations during RP-HPLC may generally be approached in two ways, mobile phase manipulation on a given reversed-phase column or employment of different stationary phases with complementary selectivities, the former approach being more commonly employed. With the wide choice of variables (*e.g.*, ion-pairing reagent, pH,

0021-9673/91/$03.50

organic modifier, other additives) available to the researcher when manipulating mobile phase conditions, in addition to the option of changing the gradient-rate and flow-rate, it is perhaps not surprising that this approach to optimization of peptide and protein separations has become dominant. However, this dominance has tended to obscure the effectiveness of employing stationary phases of different selectivities for specific applications.

Although RP-HPLC on stationary phases containing alkyl chains (*e.g.*, C_8, C_{18}) as the functional ligand is still the method of choice for most peptide [1,2] and many protein separations [3,4], less hydrophobic [5] cyanopropyl (CN) packings have been applied during RP-HPLC of both peptides and proteins [5–12]. One of the reasons why these packings have not seen more use may be due to problems of stationary phase instability [6,9,12,13], with significant and rapid loss of stationary phase ligands often observed when employing acidic mobile phases, such as the frequently used aqueous trifluoroacetic acid (TFA)–acetonitrile system (pH 2). Kirkland and co-workers [13,14] recently reported the development of stable silica-based bonded phases, based on protecting the siloxane bond between the silica and the functional ligand with bulky side groups. Monofunctional alkyl- and cyanopropylsilanes containing two isopropyl groups (instead of the usual methyl groups) were found to produce exceptionally stable alkyl and CN reversed-phase packings.

This paper compares the performances of such highly stable C_8 and CN columns during their application to RP-HPLC of a range of peptides (with varying chain length and hydrophobicity), proteins and small organic molecules. The advantages for specific applications of employing a cyanopropyl stationary phase with characteristics (in terms of hydrophobicity and selectivity) markedly different to those of the commonly used C_8 packing are clearly demonstrated.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from J. T. Baker (Philipsburg, NJ, USA) and HPLC-grade TFA from Pierce (Rockford, IL, USA). Alkylphenone standards were obtained from Pierce, thioanisole from Aldrich (Milwaukee, WI, USA), dithiothreitol (DTT) from Schwarz-Mann Biotech (Cleveland, OH, USA) and bovine ribonuclease A, equine cytochrome *c*, chicken lysozyme, papain, bovine serum albumin, bovine α-lactalbumin, sperm whale myoglobin, bovine α-chymotrypsinogen A and baker's yeast enolase from Sigma (St. Louis, MO, USA). Rabbit skeletal troponin T, troponin I and troponin C and rabbit cardiac tropomyosin were prepared from tissue extracts.

Peptide synthesis

The peptides described were synthesized either on a Beckman (Berkeley, CA, USA) Model 990 peptide synthesizer or an Applied Biosystems (Foster City, CA, USA) Model 430A peptide synthesizer, using the general procedure for solid-phase peptide synthesis described by Hodges and co-workers [15,16].

Apparatus

The HPLC instrument consisted of an HP1090 liquid chromatograph (Hewlett-

Packard, Avondale, PA, USA), coupled to an HP1040A detection system, HP9000 Series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7440A plotter.

Columns

Three columns were employed: (1) silica-based C_8 packing (150 × 4.6 mm I.D. column) containing a monomeric stationary phase of diisopropyl-*n*-octyl groups (5-μm particle size, 94-Å pore size, 5.78% carbon loading) from DuPont (Wilmington, DE, USA) (this packing is equivalent to the commercial 25-cm Zorbax-Rx columns available from MacMod Analytical, Chadds Ford, PA, USA); (2) silica-based CN packing (Zorbax SB-300CN) (150 × 4.6 mm I.D. column) containing a monomeric stationary phase of diisopropyl-3-cyanopropyl groups (6 μm, 250 Å) from Rockland Technologies (West Chester, PA, USA); and (3) Aquapore RP-300 C_8 (220 × 4.6 mm I.D. column) (7 μm, 300 Å) from Chromatographic Specialties (Brockville, Ontario, Canada). All packings and columns were new to ensure a fair comparison.

RESULTS AND DISCUSSION

Comparison of C_8 and CN column performance in RP-HPLC of peptides

Although CN packings have been employed for RP-HPLC of peptides [5–9,11,12], it has been reported [6,8,17] that hydrophilic/moderately hydrophobic and/or small peptides are generally not retained well by such packings. In contrast, such packings have proved useful for the separation of hydrophobic peptides [10,12].

The peptides shown in Table I were subjected to a linear A–B gradient (1%

TABLE I

PEPTIDES USED IN THIS STUDY

Peptides	Sequence[a]
10G–40G	Ac–(Gly–Lys–Gly–Leu–Gly)$_n$–amide, where n = 2,4,6,8; 10G, 20G, 30G, 40G, respectively
5A–50A	Ac–(Leu–Gly–Leu–Lys–Ala)$_n$–amide, where n = 1,2,4,6,8,10; 5A, 10A, 20A, 30A, 40A, 50A, respectively
5L–50L	Ac–(Leu–Gly–Leu–Lys–Leu)$_n$–amide, where n = 1,2,4,6,8,10; 5L, 10L, 20L, 30L, 40L, 50L, respectively
7A–35A	Ac–Lys–Cys–Ala–Glu–Gly–Glu–Leu–(Lys–Leu–Glu–Ala–Gly–Glu–Leu)$_n$–amide, where n = 0,1,2,3,4; 7A, 14A, 21A, 28A, 35A, respectively
7B–35B	Ac–Lys–Cys–Ala–Glu–Leu–Glu–Gly–(Lys–Leu–Glu–Ala–Leu–Glu–Gly)$_n$–amide, where n = 0,1,2,3,4; 7B, 14B, 21B, 28B, 35B, respectively
8W–8Y	Ac–Gly–X–X–(Leu)$_3$–(Lys)$_2$–amide, where X = Trp,Phe,Leu,Ile,Val,Tyr; 8W, 8F, 8L, 8I, 8V, 8Y, respectively
S1	Arg–Gly–Ala–Gly–Gly–Leu–Gly–Leu–Gly–Lys–amide
S2–S5	Ac–Arg–Gly–X–Y–Gly–Leu–Gly–Leu–Gly–Lys–amide, where X – Y = Gly–Gly, Ala–Gly, Val–Gly, Val–Val; S2, S3, S4, S5, respectively
E1	Ac–Glu–Tyr–Gly–Ala–Gly–Gly–Ala–Gly–Gly–Leu–Glu–amide
E2	Ac–Gly–Gly–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–Glu–amide
E3	Ac–Glu–Tyr–Ala–Ala–Glu–Ala–Ala–Glu–Gly–Leu–Glu–amide
E4	Ac–Gly–Gly–Ala–Leu–Glu–Ala–Ala–Glu–Gly–Leu–Glu–amide

[a] Ac denotes N^{α}-acetyl; amide denotes C^{α}-amide.

B/min at a flow-rate of 1 ml/min, where solvent A is 0.05% aqueous TFA and solvent B is 0.05% TFA in acetonitrile; pH 2) on the CN packings. As a comparison, the peptides were also chromatographed on the DuPont C_8 column. These peptides exhibit a wide range of both peptide size and hydrophobicity.

The retention data (Table II) obtained from three sets of peptides shown in Table I (the G, A and L series of peptide polymers) serve to summarize the general trend of RP-HPLC retention behaviour exhibited by all of the peptides used. For the purpose of this study, each peptide in these three series is referred to by a number and letter which denote, respectively, the number of residues it contains and to which polymer series it belongs. Thus, 10G refers to the ten-residue G series peptide, 30A refers to the 30-residue A series peptide, etc. These three sets of peptide polymers cover a similar range of chain length but differ in overall hydrophobicity; the hydrophobicity of these series of peptides increases in the order G < A < L. From Table II, each peptide was consistently eluted earlier from the CN column than from the C_8 column. Interestingly, the CN column provided greater selectivity for earlier eluted peptides. For instance, the difference in retention times (Δt) for G10 and G20 on the column was 7.4 min; in contrast, Δt for these peptides was only 3.5 min on the C_8 column. Similarly, for other peptide pairs, Δt between A5 and A10 was 14.6 min (CN) or 8.3 min (C_8), Δt between A10 and A20 was 7.9 min (CN) and 5.2 min (C_8), Δt

TABLE II

COMPARISON OF PEPTIDE RETENTION TIMES AND PEAK WIDTHS ON C_8 AND CN COLUMNS IN RP-HPLC

Peptide	C_8[a]		CN[a]	
	t_R (min)[b]	W (min)[c]	t_R (min)	W (min)
G10	20.4	0.14	7.9	0.26
G20	23.9	0.17	15.3	0.25
G30	25.4	0.14	18.5	0.20
G40	27.1	0.12	21.2	0.17
A5	24.0	0.15	8.6	0.38
A10	32.3	0.15	23.2	0.27
A20	37.5	0.15	31.1	0.22
A30	40.8	0.15	34.3	0.18
A40	43.9	0.14	37.8	0.17
A50	49.6	0.24	43.4	0.19
L5	30.8	0.16	18.1	0.24
L10	39.8	0.15	32.2	0.17
L20	45.7	0.13	39.5	0.14
L30	49.3	0.15	43.1	0.14
L40	52.2	0.20	45.8	0.15
L50	53.7	0.25	47.4	0.13

[a] C_8 denotes DuPont sterically protected C_8 column (150 × 4.6 mm I.D.); CN denotes sterically protected Zorbax SB-300CN column (150 × 4.6 mm I.D.).

[b] t_R denotes retention time; the data were obtained by linear A–B gradient elution (1% B/min and 1 ml/min), where eluent A was 0.05% aqueous TFA and eluent B was 0.05% TFA in acetonitrile (pH 2). Absorbance at 210 nm.

[c] W denotes peak width at half-height.

between L5 and L10 was 14.1 min (CN) and 9.0 min (C_8) and Δt between G20 and A5 was 6.7 min (CN) and 0.1 min (C_8).

The improvement in separation of the more hydrophilic peptides on the CN column compared with the C_8 column was achieved at the expense of some peak broadening of these peptides on the former column compared with the latter. In fact, this points to an interesting difference between the two column packings. Thus, on the less hydrophobic CN packing, peak widths are decreased, up to a point, with increasing peptide retention time, *e.g.*, the peak widths of the G series peptides decreased from 0.26 min (G10) to 0.17 min (G40) as the peptide retention time increased from 7.9 min (G10) to 21.2 min (G40). Table II shows similar results for the earlier eluted ($<$ *ca.* 35 min) A and L series peptides. In contrast to the CN column, the C_8 column exhibited the opposite peak width *vs.* retention time relationship, *i.e.*, the later eluted peaks ($>$ *ca.* 40 min) on the C_8 column exhibited some peak broadening; *e.g.*, the peak widths of L30, L40 and L50 (retention times of 49.3, 52.2 and 53.7 min, respectively) were 0.15, 0.2 and 0.25 min, respectively. Finally, it should be noted that the minimum peak widths on both columns were essentially identical, *i.e.*, *ca.* 0.12–0.15 min.

The possibility that gradient delay time (the time for the gradient to reach the top of the column from the solvent mixer) may have played a role in the observed retention behaviour of early eluted peptides on the CN column (improved separation over that observed on the C_8 column with concomitant peak broadening) was investigated. A large gradient delay time may potentially cause the separation of more hydrophilic solutes initially to be essentially isocratic. For instance, a delay time of 5 min with a mobile phase flow-rate of 1 ml/min means that 5 ml (*i.e.*, *ca.* two column volumes for a column of 150 × 4.6 mm I.D.) of starting eluent will have passed through the column, following sample injection, prior to the start of the gradient. Fig. 1 demonstrates the effect of increasing gradient delay time on the elution profile of four eleven-residue peptides, E1–E4 (Table I) on the CN column (A, B and C) and the Zorbax C_8 column (D). These N-terminal acetylated peptides contain only acidic residues (Glu) with no basic residues present, *i.e.*, the peptides are uncharged at pH 2.0, thus avoiding the complication of any potential ionic interactions with the column packings. Fig. 1A shows the elution profile of the peptides on the CN column, where the acetonitrile gradient was started immediately following sample injection. As observed previously for the earlier eluted G, A and L series peptides (Table II), the peak width decreased with increasing peptide retention time (from a peak width of 0.21 min for E1 at a retention time of 12.5 min to a peak width of 0.15 min at a retention time of 19.7 min for E4). The gradient delay time for the HP instrumentation employed in this study is low (*ca.* 2 min at a flow-rate of 1 ml/min, *i.e.*, less than one column volume for a 150 × 4.6 mm I.D. column), suggesting any potential effects of such a delay would be minimal. Fig. 1B and C show the effect of increasing the gradient delay times by a further 10 and 20 min, respectively, by employing an isocratic hold prior to the start of the gradient. These delay times translate into *ca.* five and nine column volumes (10- and 20-min isocratic holds, respectively; Fig. 1B and C, respectively) of starting eluent prior to the start of the gradient. It is immediately apparent that the increasing delay time resulted in increasing peak broadening of the earliest eluted peptide, E1. Thus, the peak width of this peptide increased from 0.21 min in the absence of an isocratic hold (Fig. 1A) to 0.34 min and, finally, 0.43 min

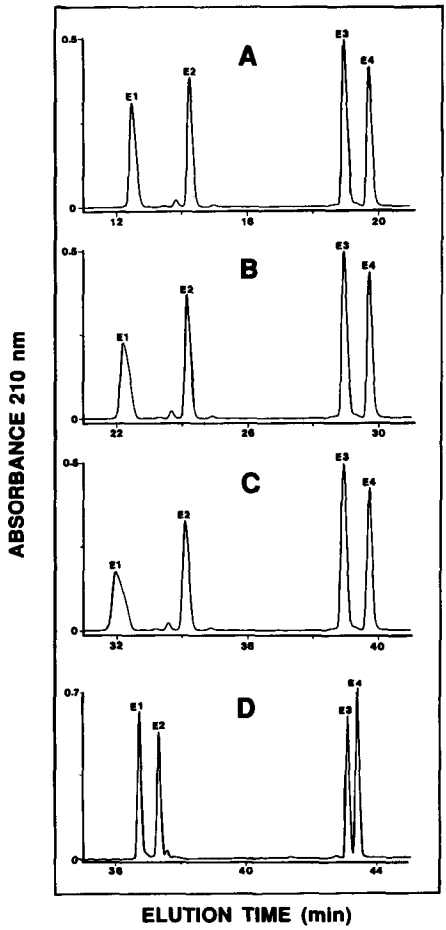

Fig. 1. Effect of gradient delay time on performance of C_8 and CN columns during RP-HPLC of peptides. Columns: sterically protected C_8 column from DuPont (150 × 4.6 mm I.D., particle size 5 μm, pore size 94 Å) and sterically protected Zorbax CN column (150 × 4.6 mm I.D.; 6 μm, 250 Å). Conditions: linear A–B gradient (1% B/min) at a flow-rate of 1 ml/min, where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA in acetonitrile (pH 2); 26°C. Absorbance at 210 nm. Sample volume: 20 μl of eluent A. (A, B and C) separation of peptides on CN column with isocratic holds (prior to the start of the acetonitrile gradient) of 0, 10 and 20 min, respectively; (D) separation of peptides on C_8 column with a 20-min isocratic hold. The sequences of peptides E1–E4 are shown in Table I.

following the employment of isocratic holds of 10 and 20 min, respectively (Fig. 1B and C, respectively). Some minor peak broadening with increasing delay time is also apparent for peptide E2, with peptides E3 and E4 unaffected. However, although Fig. 1 demonstrates that large gradient delays affected the peak broadening of the relatively hydrophilic peptide, E1, it also clearly shows that the relative separation of the four peptides was unaffected, *i.e.*, the concentration of acetonitrile required to elute each peptide remained identical in the absence or presence of an isocratic hold. Hence, the initial isocratic elution prior to the gradient affected only the peak width and not column selectivity.

Fig. 1D shows the elution profile of the four peptides on the Zorbax C_8 column following a 20-min isocratic hold. This profile was identical with that obtained in the absence of an isocratic hold, indicating that the initial isocratic elution prior to the gradient has no effect on peak broadening on this column. In a similar manner to that observed above (Table II) for the earlier eluted G, A and L series peptides, the selectivity of the CN column for peptides E1–E4 (Fig. 1A) is superior to that of the C_8 column (Fig. 1D).

Overall, the peak broadening observed on the CN column for ealier eluted peptides is no more a significant disadvantage of the column than the similar peak broadening observed for later eluted peptides on the C_8 column.

Application of the CN column to the analysis and purification of synthetic peptides

Fig. 2 compares the separation of two peptides, 8W and 8F, on (A) the C_8 and (B) the CN columns. These two peptides differ by only two residues: two phenylalanine residues in 8F are replaced by two tryptophan residues in 8W. Fig. 2 demonstrates a marked difference in their retention behaviour on the CN and C_8 columns. Thus, from a poor separation on the C_8 column (Δt = 0.3 min only) (A), the peptides were separated by a much wider margin of 4.7 min on the CN column (B). This dramatic change in the separation of the two peptides between the C_8 and CN columns implies significant selectivity differences between the dipolar cyanopropyl and the octyl functionalities. This selectivity difference may be very useful in multiple peptide synthesis [18], a method to synthesize, cleave and purify several peptides simultaneously in a single batch. Up to now, the limiting factor in the success of this technique has been the resolving power of HPLC for resolving complex peptide mix-

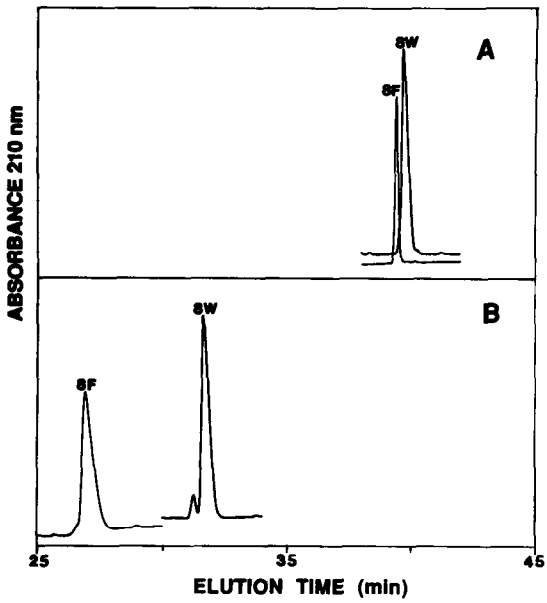

Fig. 2. RP-HPLC of synthetic peptides on (A) C_8 and (B) CN columns. Columns and conditions as in Fig. 1. The sequences of peptides 8F and 8W are shown in Table I.

tures quickly and efficiently [18]. An important practical feature of the sterically protected CN column in such applications is the outstanding stability of this ligand in aggressive environments. It should also be noted that peptides which are eluted together on the CN column may be separated on the C_8 column.

Fig. 3 compares the chromatographic behaviour of peptides with that of a series of three alkylphenones (propiophenone, butyrophenone and valerophenone; A2, A3 and A4, respectively) on (A) the C_8 and (B) the CN columns. The alkylphenones, commonly used as internal standards for RP-HPLC, were chosen to represent typical small organic molecules.

All of the four peptides shown in Fig. 3 exhibited similar elution behaviour on the two columns in terms of both retention time and satisfactory peak shape. In contrast, the alkylphenones showed a dramatic difference in their retention behaviour on the two columns. On the C_8 column (Fig. 3A), the alkylphenones were retained to an extent similar to that of the four peptides, making the resolution of these two classes of compounds difficult. In contrast, on the CN column (Fig. 3B), the alkylphenones were barely retained, greatly simplifying their separation from the peptides. In addition, it should be noted that peptides 21B and 35A were much better resolved on the CN column (Fig. 3B) than on the C_8 column (Fig. 3A) (there was, in fact, a reversal of elution order), again implying selectivity differences between the two types of functional groups. DTT (dithiothreitol) was added to the sample mixture to keep peptides 35A, 21B and 35B (Table I) in their reduced form and, hence, prevent interchain disulphide bond formation between peptides.

As noted above (Table II, Figs. 1–3), the wide variation in retention behaviour

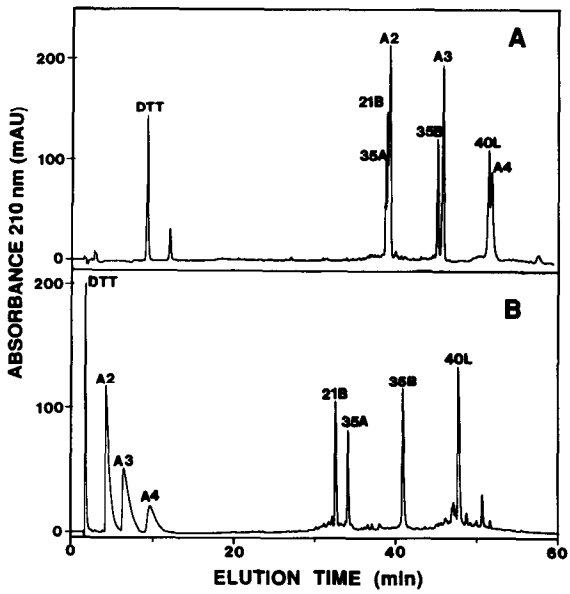

Fig. 3. RP-HPLC of mixtures of synthetic peptides and small organic molecules on (A) C_8 and (B) CN columns. Columns and conditions as in Fig. 1. The sequences of peptides 35A, 21B, 35B and 40L are shown in Table I. A2, A3 and A4 denote propiophenone, butyrophenone and valerophenone, respectively; DTT denotes dithiothreitol.

for alkylphenones and peptides on the C_8 and CN columns is illustrative of the different selectivities associated with these ligands. This selectivity difference is a function of two properties. First, the more polar CN phase itself can exhibit significantly different interactions with solutes, relative to those with the highly hydrophobic, less polar C_8 phase. Second, the level of organic modifier needed for solute elution is significantly less for the CN phase than the C_8 phase; the CN phase is much weaker [19]. It is well known that the selectivity of reversed-phase separations often is strongly affected by the percentage of organic component or the concentration of water associated with elution [20]. Stated otherwise, the log *k' versus* percentage organic component plots for different solutes often show different slopes. For weaker phases such as CN, lower concentrations of organic component (higher concentrations of water) are required, compared with C_8 phases, creating an environment for significant potential changes in band spacing. This is especially the case for mixtures of small and large molecules that also exhibit profound differences in the intercepts of log *k' versus* percentage organic component plots (plots for peptides are much steeper than those for alkylphenones). Therefore, striking differences in the log *k' versus* percentage organic component relationships often lead to large differences in band spacing, such as exhibited for the alkylphenone/peptide data in Fig. 3. Changing from the strongly hydrophobic C_8 phase to CN creates an environment whereby such selectivity differences are greatest. The band-spacing differences seen in Fig. 3 are further enhanced as a result of the lower surface area of the CN column (wider pores) compared with the C_8 column. This magnifies the effect of column strength, as even less organic component (more water) is required to elute the same components.

The results shown in Fig. 3 suggested a role for the CN column in purifying organic contaminants, such as those encountered in solid-phase peptide synthesis. These contaminants, including side-chain protecting groups, coupling reagents, cleavage reagents and scavengers, are often difficult to separate from the desired peptide product during RP-HPLC on C_8 and C_{18} columns. For example, thioanisole is a good scavenger and accelerator of the reaction cleaving the synthesized peptide from the resin support [21,22]. In many cases, this scavenger is eluted with the peptide product of interest from an alkyl-bonded reversed-phase column. An example of this can be seen in Fig. 4A, where thioanisole (T) was not separated from synthetic peptide 35B on the C_8 column (the DTT is again present to prevent interchain disulphide bond formation between peptide molecules). In contrast, on the CN column (Fig. 4B), whereas the retention time of the peptide was similar to that exhibited on the C_8 column (Fig. 4A), thioanisole was now barely retained, thus achieving an easy separation.

Purification of extremely hydrophobic peptides on the CN column

Very hydrophobic peptides, such as membrane-associated peptides, often pose special problems during RP-HPLC owing to their limited solubility and tendency to aggregate. In addition, they may be adsorbed irreversibly to some reversed-phase sorbents [23]. Gerber *et al.* [24] and Takagaki *et al.* [25] successfully separated hydrophobic peptides by RP-HPLC with a mobile phase consisting of formic acid, water and ethanol. The difficulty with this system is that peptide detection by UV absorbance is only possible at relatively insensitive wavelengths such as 280 nm, owing to the presence of formic acid. Knighton *et al.* [12] employed RP-HPLC with a mobile

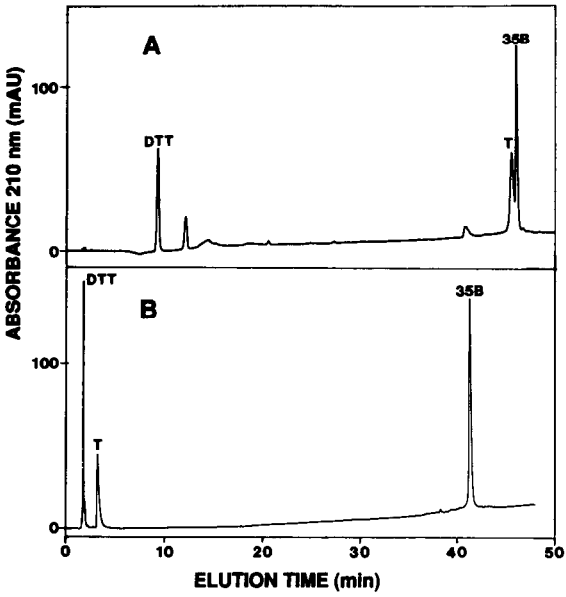

Fig. 4. RP-HPLC of synthetic peptide and thioanisole (T) on (A) C_8 and (B) CN columns. Columns and conditions as in Fig. 1. The sequence of peptide 35B is shown in Table I. DTT denotes dithiothreitol.

phase containing ammonium hydrogencarbonate to purify lipid-associated peptides successfully. However, it was very difficult to work with this mobile phase owing to the formation of carbon dioxide bubbles in the detector flow cell [12]. Tomich *et al.* [26] recently reported that the addition of the non-ionic detergent *n*-octyl-β-D-glucopyranoside to the mobile phase can prevent membrane-spanning peptides from binding irreversibly to a reversed-phase packing. This detergent binds to the stationary phase, reducing the potential sites of interaction on the solid matrix [26]. When employing this mobile phase, a subsequent dialysis step is required to remove the detergent from the eluted peptides. Taneja *et al.* [27] reported the separation of hydrophobic peptide polymers on a C_3 column through employment of 2-propanol as the organic modifier. Despite these reports of successful RP-HPLC purification of exceptionally hydrophobic peptides, routine methods for such purifications are not yet well established.

The major problem limiting routine successful purification of very hydrophobic peptides by RP-HPLC is the excessive strength of hydrophobic interaction between the peptides and alkyl-bonded stationary phases such as C_8 and C_{18} sorbents. It seemed reasonable, therefore, that a less hydrophobic stationary phase, such as the CN stationary phase, may be more promising for this kind of application. Fig. 5 compares the elution profile of a hydrophobic synthetic peptide P22 [Ac–$(Lys)_2$–Gly–$(Leu)_{16}$–$(Lys)_2$–Ala–amide] on the C_8 (Fig. 5A) and CN (Fig. 5B) columns. About 12% less acetonitrile in the mobile phase was required to elute P22 from the CN column compared with the C_8 column. In addition, a greater separation of P22 from impurities (I) was achieved on the CN column. Finally, the peak width of P22 on the CN column was less than that on the C_8 column (0.32 and 0.50 min, respectively). Similar results were obtained with synthetic peptide P26 [Ac–$(Lys)_2$–Gly–$(Leu)_{20}$–

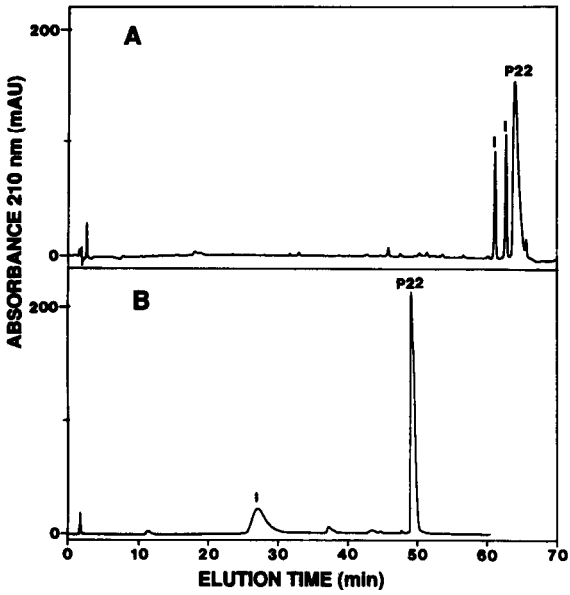

Fig. 5. RP-HPLC of a synthetic hydrophobic peptide on (A) C_8 and (B) CN columns. Columns and conditions as in Fig. 1, except sample volume is 20 μl in 70% eluent A–30% eluent B. The sequence of peptide P22 is shown in the text. I denotes impurity.

$(Lys)_2$–Ala–amide], with peak widths of 0.52 and 0.38 min on the C_8 and CN columns, respectively. These results, coupled with those presented in Fig. 1, suggest that the employment of a CN column with an aqueous TFA–acetonitrile mobile phase is a simple and effective method for purification of very hydrophobic peptides.

RP-HPLC of proteins on the C_8 and CN columns

There has been a significant increase in recent years in the application of RP-HPLC to the analysis and separation of proteins [4,28]. The best recovery and overall resolution of proteins has generally been demonstrated on large-pore, silica-based stationary phases containing relatively short alkyl chains (*e.g.*, C_3) [29,30]. The trade-off has been that such short-chain stationary phases (and, indeed, cyano phases) are generally less stable than those with longer alkyl ligands, which shield more effectively the underlying siloxane bonds from hydrolysis by the mobile phase. However, this problem is minimized or even eliminated with such sterically protected packings as those employed in the present study and described previously [13].

Table III compares the performances of the sterically protected C_8 (C_8-2 in Table III) and CN columns during the RP-HPLC of thirteen proteins with that of a conventional C_8 packing containing dimethyloctyl groups (C_8-1 in Table III). The latter C_8 column was chosen because in our hands it has proved to be one of the better commercially available packings. The peak width at half-height was again used as an index of column performance. All three columns were run under identical linear A–B gradient elution conditions (1%B/min and 1 ml/min), where eluent A was 0.05% aqueous TFA and eluent B was 0.05% TFA in acetonitrile (pH 2).

From Table III, it is clearly apparent that the peak widths of all thirteen proteins were significantly less on the sterically protected C_8 packing (C_8-2) than on the conventional C_8 column (C_8-1), even though the pore size of the former column is only 94 Å compared with 300 Å for the latter. The smaller particle size of the sterically protected packing (5 μm) compared with the conventional packing (7 μm) may partly explain the narrower peptide peak widths on the former column, although the relatively small difference in these particle sizes probably had no major effect. With the exception of ribonuclease A, the peak widths of the proteins were even smaller on the sterically protected CN column compared with the DuPont C_8 column (C_8-2).

The CN column also exhibited the best column performance of the three in terms of protein resolution. Based on the data shown in Table III, the resolution (R_s) of any two peaks was calculated according to the equation

$$R_s = \frac{1.176\Delta t}{W_1 + W_2}$$

where Δt is the difference in retention time between two protein peaks (1 and 2) and W_1 and W_2 are their peak widths at half-height. The resolution of every possible combination of protein pairs within the thirteen proteins was calculated for all three columns. Out of a total of 78 possible combinations of protein pairs, only five calculated resolutions were less on the CN column than on the DuPont C_8 column (C_8-2).

TABLE III

COMPARISON OF PROTEIN RETENTION TIMES AND PEAK WIDTHS ON C_8 AND CN COLUMNS IN RP-HPLC

No.	Protein	N[a]	C_8-1[b]		C_8-2[b]		CN[b]	
			t_R (min)[c]	W (min)[d]	t_R (min)	W (min)	t_R (min)	W (min)
1	Ribonuclease	124	32.4	0.44	28.7	0.30	24.0	0.38
2	Troponin T	259	39.3	0.78	33.7	0.34	31.1	0.15
3	Cytochrome *c*	104	37.8	0.50	34.0	0.32	30.4	0.23
4	Lysozyme	129	40.8	0.42	36.8	0.30	33.3	0.25
5	Troponin I	178	42.2	0.68	37.2	0.39	34.9	0.19
6	Bovine serum albumin	582	44.5	0.81	40.4	0.60	36.1	0.50
7	Papain	212	45.9	1.12	40.6	0.65	37.6	0.29
8	α-Lactalbumin	123	45.0	0.46	41.0	0.44	35.9	0.18
9	Tropomyosin	284	46.9	1.12	41.7	0.79	39.3	0.46
10	Myoglobin	153	48.0	0.77	42.5	0.38	38.4	0.17
11	α-Chymotrypsinogen A	245	48.4	0.58	42.7	0.38	39.7	0.19
12	Enolase	436	52.1	0.77	45.5	0.35	42.4	0.29
13	Troponin C	159	52.6	0.49	47.8	0.39	44.3	0.19

[a] N denotes number of amino acid residues.

[b] C_8-1 denotes Aquapore RP-300 C_8 column (220 × 4.6 mm I.D.); C_8-2 denotes DuPont sterically protected C_8 column (150 × 4.6 mm I.D.); CN denotes Zorbax sterically protected cyanopropyl column (150 × 4.6 mm I.D.) (see Experimental).

[c] t_R denotes retention time; the data were obtained by linear A–B gradient elution (1% B/min and 1 ml/min), where eluent A was 0.05% aqueous TFA and eluent B was 0.05% TFA in acetonitrile (pH 2). Absorbance at 210 nm.

[d] W denotes peak width at half-height.

In addition, 24 out of the possible 78 combinations showed an increase in protein resolution of $>100\%$ compared with this C_8 column (C_8-2).

Fig. 6 demonstrates the elution profiles of selected mixtures of proteins on (A and C) the DuPont C_8 and (B and D) the Zorbax CN columns. It is apparent from Fig. 6A and B that the columns exhibited considerable selectivity differences for some proteins. Thus, on the C_8 column (Fig. 6A), protein pairs of bovine serum albumin and papain (proteins 6 and 7 in Table III) and myoglobin and α-chymotrypsinogen A (proteins 10 and 11) were completely unresolved. In contrast, all four proteins were resolved on the CN column (Fig. 6B). Ferris *et al.* [31] made a similar observation concerning selectivity differences between C_3, diphenyl and CN columns during RP-HPLC of ribosomal proteins.

From Fig. 6C and D, the CN column performance (D) is clearly superior to that of the C_8 column (C) for the mixture of five proteins (troponin T, troponin I, α-lactalbumin, myoglobin and troponin C; proteins 2, 5, 8, 10 and 13, respectively), although the separation of specific proteins may be superior on the C_8 column, *e.g.*, note the improved separation of troponin I (protein 5) from α-lactalbumin (protein 8) on the C_8 column (Fig. 6C) compared with the CN column (Fig. 6D). The overall superior performance of the CN column is exemplified by both narrower peak widths and milder elution conditions (*i.e.*, less organic solvent) required to elute the proteins from this less hydrophobic packing. Milder elution conditions frequently translate into better recoveries of purified proteins compared with those obtained from more hydrophobic hydrocarbon stationary phases [32].

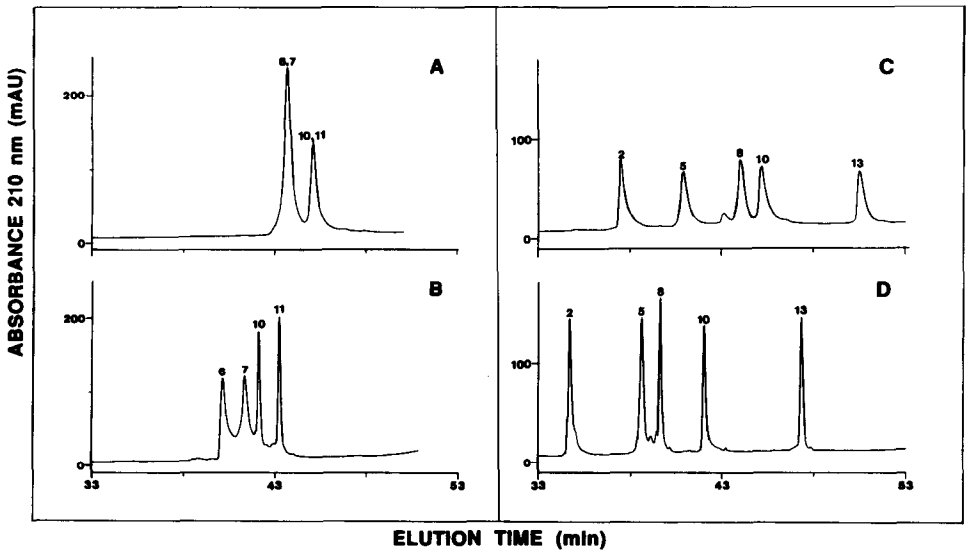

Fig. 6. RP-HPLC of proteins on (A and C) C_8 and (B and D) CN columns. Columns and conditions as in Fig. 1. Peak numbers denotes proteins shown in Table III.

CONCLUSIONS

Comparison of C_8 and CN column performance during peptide and protein separations clearly show how stationary phases of different hydrophobicities and selectivities may complement each other for specific applications. In addition, the results suggest that optimization of peptide and protein separations through employment of different RP-HPLC stationary phases (as opposed to the more common optimization protocol of manipulating the mobile phase) is an approach underestimated in the past.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada and by equipment grants, a postdoctoral fellowship (N.E.Z.) and a research allowance from the Alberta Heritage Foundation for Medical Research.

REFERENCES

1 C. T. Mant and R. S. Hodges, in K. Gooding and F. E. Regnier (Editors), *HPLC of Biological Macromolecules: Methods and Applications*, Marcel Dekker, New York, 1990, p. 301.
2 C. T. Mant, N. E. Zhou and R. S. Hodges, in E. Heftmann (Editor), *Chromatography, Part B*, Elsevier Amsterdam, 5th ed., in press.
3 F. E. Regnier, *Methods Enzymol.*, 91 (1983) 137.
4 K. Gooding and F. E. Regnier (Editors), *HPLC of Biological Macromolecules: Methods and Applications*, Marcel Dekker, New York, 1990.
5 C. T. Wehr, L. Correia and S. R. Abbott, *J. Chromatogr. Sci.*, 20 (1982) 114.
6 I. M. Chaiken and C. J. Hough, *Anal. Biochem.*, 107 (1980) 11.
7 M. A. Anzano, A. B. Roberts, J. M. Smith, L. C. Lamb and M. B. Sporn, *Anal. Biochem.*, 125 (1982) 217.
8 D. M. Abercrombie, C. J. Hough, J. R. Seeman, M. J. Brownstein, H. Gainer, J. T. Russell and I. M. Chaiken, *Anal. Biochem.*, 125 (1982) 395.
9 D. R. Knighton, D. R. K. Harding, J. R. Napier and W. S. Hancock, *J. Chromatogr.*, 249 (1982) 193.
10 G. E. Tarr and J. W. Crabb, *Anal. Biochem.*, 131 (1983) 99.
11 P. Tempst, M. W. Hunkapiller and L. E. Hood, *Anal. Biochem.*, 137 (1984) 188.
12 D. R. Knighton, D. R. K. Harding, J. R. Napier and W. S. Hancock, *J. Chromatogr.*, 347 (1985) 237.
13 J. J. Kirkland, J. L. Glajch and R. D. Farlee, *Anal. Chem.*, 61 (1988) 2.
14 J. L. Glajch and J. J. Kirkland, *US Pat.*, 4 705 725, (1988).
15 J. M. R. Parker and R. S. Hodges, *J. Protein Chem.*, 3 (1985) 465.
16 R. S. Hodges, P. D. Semchuk, A. K. Taneja, C. M. Kay, J. M. R. Parker and C. T. Mant, *Pept. Res.*, 1 (1988) 19.
17 B. Fransson, *J. Chromatogr.*, 361 (1986) 161.
18 F. S. Tjoeng, D. S. Towery, J. W. Bulock, D. E. Whipple, K. F. Fok, M. H. Williams, M. E. Zupec and S. P. Adams, *Int. J. Pept. Protein Res.*, 35 (1990) 141.
19 P. E. Antle, A. P. Goldberg and L. R. Snyder, *J. Chromatogr.*, 321 (1985) 1.
20 L. R. Snyder and P. E. Antle, *LC · GC*, 3 (1985) 98.
21 H. Yajima, N. Fujii, S. Funakoshi, T. Watanabe, E. Murayama and A. Otaka, *Tetrahedron*, 44 (1988) 805.
22 J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, Pierce, Rockford, IL, 1985, p. 38.
23 C. Edelstein and A. M. Scanu, in W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1984, p. 405.
24 G. E. Gerber, R. J. Anderegg, W. C. Herlihy, C. P. Gray, K. Biemann and H. G. Khorana, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 227.
25 Y. Takagaki, G. E. Gerber, K. Nihei and H. G. Khorana, *J. Biol. Chem.*, 255 (1980) 1536.

26 J. M. Tomich, L. W. Carson, K. J. Kanes, N. J. Vogelaar, M. R. Emerling and J. M. Richards, *Anal. Biochem.*, 174 (1988) 197.
27 A. K. Taneja, S. Y. M. Lau and R. S. Hodges, *J. Chromatogr.*, 317 (1984) 1.
28 C. T. Mant and R. S. Hodges (Editors), *HPLC of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, 1991.
29 J. D. Pearson and F. E. Regnier, *J. Liq. Chromatogr.*, 6 (1983) 497.
30 J. A. Bietz, in K. Gooding and F. E. Regnier (Editors), *HPLC of Biological Macromolecules: Methods and Applications*, Marcel Dekker, New York, 1990, p. 429.
31 R. J. Ferris, C. A. Cowgill and R. R. Traut, *Biochemistry*, 23 (1984) 3434.
32 M. Hermodson and W. C. Mahoney, *Methods Enzymol.*, 91 (1983) 352.