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Hydrophilic-interaction chromatography of peptides on hydrophilic and strong cation-exchange columns

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ABSTRACT

Hydrophilic-interaction chromatography (HILIC) was recently introduced as a potentially useful separation mode for the purification of peptides and other polar compounds. The elution order of peptides in HILIC, which separates solutes based on hydrophilic interactions, should be opposite to that obtained in reversed-phase chromatography, which separates solutes based on hydrophobic interactions. Three series of peptides, two of which consisted of positively charged peptides (independent of pH at pH < 7) and one of which consisted of uncharged or negatively charged peptides (dependent on pH), and which varied in overall hydrophilicity/hydrophobicity, were utilized to examine the separation mechanism and efficiency of HILIC on hydrophilic and strong cation-exchange columns.

INTRODUCTION

Hydrophilic-interaction chromatography (HILIC) has recently been promoted as a novel chromatographic mode for application to the separation of a wide range of solutes [1]. Separation by HILIC, in a manner similar to normal-phase chromatography (to which it is related), depends on hydrophilic interactions between the solutes and the hydrophilic stationary phase, *i.e.*, solutes are eluted from a HILIC column in order of increasing hydrophilicity (decreasing hydrophobicity). Thus, elution orders of solutes in HILIC should be opposite to that obtained in reversed-phase high-performance liquid chromatography (RP-HPLC), which separates solutes based on hydrophobic interactions, *i.e.*, solutes are eluted from a RP-HPLC column in order of increasing hydrophobicity (decreasing hydrophilicity). HILIC is characterized by separations being effected by a linear A–B gradient of decreasing organic modifier concentration, *i.e.*, starting from a high concentration of organic modifier (typically, 70–90% aqueous acetonitrile).

In this study, we set out to evaluate the potential of HILIC for peptide separations. To this end, we examined the retention behavior of three series of model synthetic peptides varying in both hydrophobicity/hydrophilicity and charge. From the observed retention behavior of these series of model peptides (encompassing various controlled combinations of peptide characteristics) during HILIC on both HILIC and strong cation-exchange columns, we were able to assess the potential

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TABLE I

PROPERTIES OF SYNTHETIC PEPTIDE STANDARDS

Peptide	Peptide sequence[a]	Net charge		Hydrophilicity/hydrophobicity[b]	
		pH 2.0	pH 6.5	pH 2	pH 7
S1	*NH_2–*Arg–Gly–**Ala–Gly**–Gly–Leu–Gly–Leu–Gly–*Lys–amide	+3	+3	11.5	19.9
S2	Ac–*Arg–Gly–**Gly–Gly**–Gly–Leu–Gly–Leu–Gly–*Lys–amide	+2	+2	12.3	17.5
S3	Ac–*Arg–Gly–**Ala–Gly**–Gly–Leu–Gly–Leu–Gly–*Lys–amide	+2	+2	14.5	19.9
S4	Ac–*Arg–Gly–**Val–Gly**–Gly–Leu–Gly–Leu–Gly–*Lys–amide	+2	+2	17.5	23.4
S5	Ac–*Arg–Gly–**Val–Val**–Gly–Leu–Gly–Leu–Gly–*Lys–amide	+2	+2	22.7	29.3
C1	Ac–Gly–Gly–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–*Lys–amide	+1	+1	14.7	18.6
C2	Ac–*Lys–Tyr–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–*Lys–amide	+2	+2	17.5	23.4
C3	Ac–Gly–Gly–Ala–Leu–*Lys–Ala–Leu–*Lys–Gly–Leu–*Lys–amide	+3	+3	21.4	30.2
C4	Ac–*Lys–Tyr–Ala–Leu–*Lys–Ala–Leu–*Lys–Gly–Leu–*Lys–amide	+4	+4	24.2	35.0
A1	Ac–†Glu–Tyr–Gly–Ala–Gly–Gly–Ala–Gly–Gly–Leu–†Glu–amide	0	−2	17.8	14.4
A2	Ac–Gly–Gly–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–†Glu–amide	0	−1	17.9	17.5
A3	Ac–†Glu–Tyr–Ala–Ala–†Glu–Ala–Ala–†Glu–Gly–Leu–†Glu–amide	0	−4	24.8	17.0
A4	Ac–Gly–Gly–Ala–Leu–†Glu–Ala–Ala–†Glu–Gly–Leu–†Glu–amide	0	−3	24.9	20.1

[a] Ac = N^{α}-Acetyl; amide = C^{α}-amide. Variations in the composition of S1–S5 are shown in bold. Potentially positively charged residues (Lys, Arg) are denoted *; potentially negatively charged residues (Glu) are denoted †.

[b] Peptide hydrophilicity/hydrophobicity is expressed as the sum of the hydrophobicity coefficients (ΣR_c) of Guo *et al.* [4].

value of HILIC for peptide separations, and also draw some conclusions concerning the mechanism of such separations.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile and reagent-grade potassium chloride were obtained from J. T. Baker (Phillipsburg, NJ, USA), HPLC-grade trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA), ACS-grade orthophosphoric acid from Anachemia (Toronto, Canada) and synthetic peptide standards for RP-HPLC (S1–S5), cation-exchange chromatography (CEC) (C1–C4) and anion-exchange chromatography (AEC) (A1–A4) from Synthetic Peptides Inc. (University of Alberta, Edmonton, Canada). The sequences of the three series of standards are shown in Table I.

Apparatus

The HPLC instrument consisted of a Spectra-Physics (San Jose, CA, USA) SP8700 solvent-delivery system, SP8750 organizer coupled to an Hewlett-Packard (Avondale, PA, USA) HP 1040A detection system, HP 3390A integrator, HP 85 computer, HP 9121 disc drive and HP 7470 plotter.

Columns

Peptides were separated on four columns: (1) a polyhydroxyethylaspartamide HILIC column, 200 × 4.6 mm I.D., particle size 5 μm, pore size 300 Å (PolyLC, Columbia, MD, USA); (2) a polysulfoethylaspartamide strong cation-exchange column, 200 × 4.6 mm I.D., 5 μm, 200 Å (PolyLC); (3) a Mono S HR 5/5 strong cation-exchange column, 50 × 5 mm I.D., 10 μm (Pharmacia, Dorval, Canada); and (4) a SynChropak RP-P C_{18} reversed-phase column, 250 × 4.6 mm I.D., 6.5 μm, 300 Å (SynChrom, Lafayette, IN, USA).

Synthetic model peptides

The relevant properties of the model peptides employed are shown in Table I. Peptide standards S1–S5 were originally designed to monitor RP-HPLC column performance (efficiency, selectivity and resolution) [2]. Each of the peptides carries a net positive charge of +3 (S1) or +2 (S2–S5) at pH between 2 and 7, *i.e.*, the pH range over which silica-based columns may be employed. Peptides C1–C4 were designed to monitor cation-exchange column performance [3]. These four peptides contain only basic residues (no acidic residues are present) and the net positive charges on the standards (+1, +2, +3 and +4 for C1, C2, C3 and C4, respectively) remain unaltered over the range pH 2–7. Peptides A1–A4 are monitors of anion-exchange column performance. The net charge on these four peptides is pH dependent (the peptides contain only acidic residues, no basic residues are present: at pH <4.0–4.5 (the pK_a of the side-chain carboxyl group of glutamic acid), the glutamic acid side-chain carboxyl is protonated, *i.e.*, uncharged; at pH >4.0–4.5, the glutamic acid side-chain carboxyl is deprotonated, *i.e.*, negatively charged. Thus, at pH 2, the four peptides are uncharged; at pH 7, peptides A1, A2, A3 and A4 possess net charges of −1, −2, −3 and −4, respectively.

A convenient means of assigning hydrophilicity/hydrophobicity values to the model peptides was required. Guo *et al.* [4] determined a precise set of amino acid side-chain hydrophilicity/hydrophobicity parameters (or coefficients) by examining the effect of different residue side-chains on the RPC retention time, during linear gradient elution at pH 2 and 7, of a synthetic model octapeptide, Ac–Gly–X–X–$(Leu)_3$–$(Lys)_2$–amide, where X was substituted by the 20 amino acids found in proteins. The overall hydrophilicity/hydrophobicity of each peptide in Table I was determined by summing the coefficients (ΣR_c) of Guo *et al.* [4] for all of the residues in the peptide.

RESULTS AND DISCUSSION

HPLC of synthetic model peptides

Fig. 1A shows the reversed-phase separation on the C_{18} column of peptides S1–S5. Aqueous TFA–acetonitrile mobile phases at pH 2 are currently employed for most peptide separations [5,6], and the separation shown in Fig. 1A was achieved by a linear A–B gradient of increasing acetonitrile concentration (1% B/min at a flow-rate of 1 ml/min), where eluent A was 0.05% aqueous TFA and eluent B was 0.05% TFA in acetonitrile. As expected, the elution order of the peptides was in order of increasing peptide hydrophobicity, with the least hydrophobic peptide (S1; ΣR_c = 11.5)

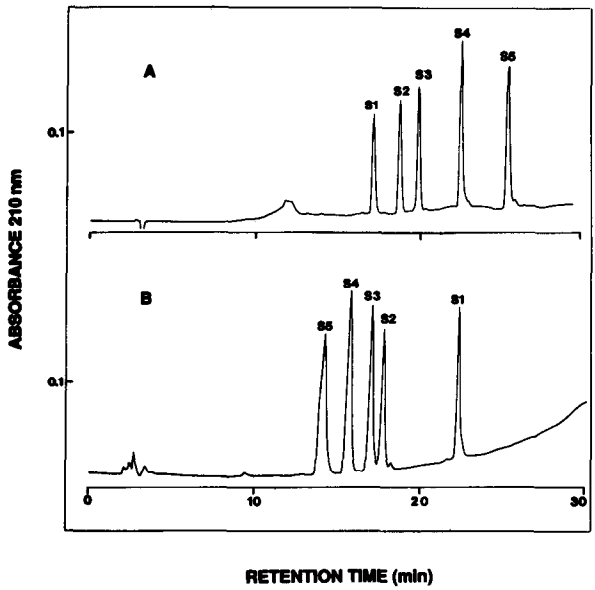

Fig. 1. Separation of a mixture of synthetic positively charged peptides by (A) RP-HPLC and (B) HILIC. (A) Column, SynChropak RP-P C_{18} (250 × 4.6 mm I.D.); mobile phase, linear A–B increasing acetonitrile gradient (1% B/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in acetonitrile; temperature, 26°C. (B) Column, Polyhydroxyethylaspartamide HILIC column (200 × 4.6 mm I.D.); mobile phase, linear A–B decreasing acetonitrile gradient (1% B/min) at a flow-rate of 1 ml/min, where A is 0.2% orthophosphoric acid in acetonitrile and B is 0.2% aqueous orthophosphoric acid (starting conditions were 85% A–15% B); temperature, 26°C. The sequences of peptides S1–S5 are shown in Table I.

being eluted first and the most hydrophobic peptide (S5; $\Sigma R_c = 22.7$) being eluted last (Table I).

Fig. 1B shows the separation of the same peptides on the HILIC column. The peptides were subjected to a linear A–B gradient of decreasing acetonitrile concentration (1% B/min, starting from 85% A–15% B, at a flow-rate of 1 ml/min) at pH 2, where eluent A was 0.2% H_3PO_4–acetonitrile and eluent B was 0.2% aqueous H_3PO_4. In RP-HPLC, hydrophobic interactions between peptides and the hydrophobic stationary phase are favoured owing to the absence of organic modifier (acetonitrile) in the starting eluent (0.05% aqueous TFA); elution of peptides is then achieved with an increasing concentration of acetonitrile in the mobile phase. In contrast, in HILIC, hydrophilic interactions between peptides and the hydrophilic stationary phase are favoured owing to the presence of a high concentration of acetonitrile in the starting eluent (85% aqueous acetonitrile–0.2% H_3PO_4); elution of peptides is then achieved with a decreasing concentration of acetonitrile in the mobile phase.

It has been observed by others [7–12] that high concentrations of organic modifier or inverse gradients (high concentrations of organic modifier decreasing to lower concentrations) on reversed-phase columns have shown elution order reversals for peptides and proteins compared with standard RP-HPLC conditions (low concentrations of organic modifier increasing to higher concentrations). Silanophilic interactions were deemed responsible for these results. Simpson and Moritz [12] recently showed that inverse gradients only show elution order reversals for some proteins and only on particular reversed-phase columns. The reversals that were observed were explained by silanophilic interactions and/or conformational changes induced in the proteins by the high concentration of organic modifier. In contrast, the results of this study with peptides showing elution order reversal on comparing RP-HPLC and HILIC (Fig. 1) is due to the hydrophobic/hydrophilic interactions of the peptides with the mobile phase/stationary phase. The peptides S2–S5 (Fig. 1) each have the same basic character (one Arg and one Lys residue) and the same net positive charge (+2 at pH 2), which rules out silanophilic interactions as a mechanism for elution order reversal.

TFA is a moderately hydrophobic anionic ion-pairing reagent [5,6,13], *i.e.*, the negatively charged trifluoroacetate ion will ion pair with positively charged groups (such as α-amino groups and the side-chains of basic residues) and, thus, increase the peptide retention time (and apparent hydrophobicity) during RP-HPLC of peptides containing these groups. In HILIC, it is desirable to emphasize peptide hydrophilicity as much as possible. Therefore, orthophosphoric acid was used in place of TFA in the HILIC mobile phase, the phosphate ion being a much more hydrophilic counter ion than trifluoroacetate [13]. From Fig. 1B, the peptides were eluted in opposite order to that obtained during RP-HPLC (Fig. 1A), as would be expected from an HILIC separation. It might be expected that the relative retention time differences between individual peptides would be approximately the same in both RP-HPLC and HILIC. However, the retention time of peptide S1 on the HILIC column was considerably longer than expected, in relation to the other four peptides, based on hydrophilicity considerations alone. This observation suggested that the HILIC column may be exhibiting ionic interactions, *i.e.*, a mixed-mode separation, based on both peptide hydrophilicity and net charge. If the HILIC sorbent possessed some negatively

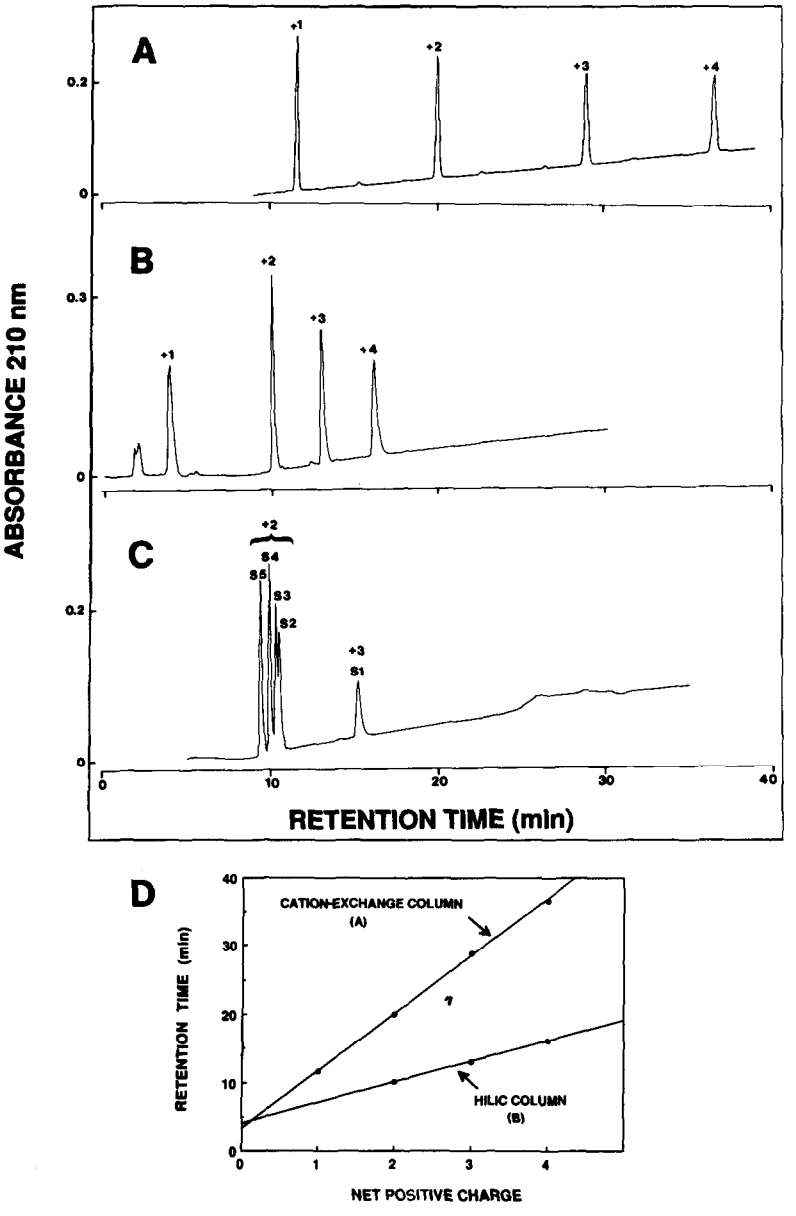

Fig. 2. Separation of a mixture of synthetic positively charged peptides by cation-exchange chromatography on (A) a strong cation-exchange column and (B and C) a HILIC column. (A) Column, Polysulfoethylaspartamide strong cation-exchange column (200 × 4.6 mm I.D.). (B and C) polyhydroxyethylaspartamide HILIC column (200 × 4.6 mm I.D.). Mobile phase: linear A–B gradient (2% B/min, equivalent to 5 m*M* KCl/min) at a flow-rate of 1 ml/min), where buffer A is 5 m*M* KH_2PO_4 (pH 6.5) and buffer B is buffer A plus 0.25 *M* KCl, both buffers containing 50% (v/v) acetonitrile; temperature, 26°C. A and B show the separation of the cation-exchange peptide standards C1, C2, C3 and C4 (+1, +2, +3 and +4 net charge, respectively). C shows the separation of peptide standards S1–S5. D shows the relationship between peptide retention time and net positive charge during cation-exchange chromatography of peptides C1–C4 on the (A) cation-exchange and (B) HILIC columns. Peptide C1 (+1 net charge) was eluted prior to the salt gradient on the HILIC column (B) and is not included in the plot in D. The sequences of the peptides are shown in Table I.

charged (*i.e.*, anionic) character, then peptide S1 (+3 net charge) would interact more strongly than peptides S2–S5, all four of which have a net charge of +2. The relative retention time difference between peptides S2, S3, S4 and S5 during HILIC are consistent with those obtained by RP-HPLC, suggesting that peptides with the same net charge exhibit similar relative hydrophilic and hydrophobic effects.

Peptide cation-exchange standards C1–C4 (+1 to +4 net charge, respectively) (Table I) were utilized to verify that the HILIC column was exhibiting ionic interactions. As noted previously, the retention behavior of these peptides should be unaffected in the pH range 2–7.

Fig. 2A and B show the elution profiles of the four peptide standards on a strong cation-exchange column (polysulfoethylaspartamide [1,14,15]) and the HILIC column, respectively. Both columns were run under standard ion-exchange conditions, *i.e.*, increasing salt (KCl) gradient in a phosphate buffer (5 m*M* aqueous KH_2PO_4, pH 6.5). Acetonitrile is often added to the mobile phase to suppress hydrophobic interactions with ion-exchange sorbents and to ensure ideal ion-exchange behavior [3]. Both the cation-exchange column (Fig. 2A) and the HILIC column (Fig. 2B) required a salt gradient to elute the peptide standards. In addition, as would be expected with ideal cation-exchange column behaviour [3], the peptides show a linear relationship between retention time and net positive charge [except peptide C1 (+1 net charge), which is eluted prior to the salt gradient on the HILIC column] (Fig. 2D). These results clearly indicated that, under these conditions, the HILIC column was behaving as a cation-exchange column, *i.e.*, the HILIC sorbent must contain negatively charged functionalities as a result of the manufacturing process. The ideal HILIC column (*i.e.*, solute separation based on hydrophilic interactions alone) would not show such ionic interactions. The shorter retention times of the peptides on the HILIC column compared with the cation-exchange column may be explained by the former possessing a smaller ion-exchange capacity.

Fig. 2C shows the elution profile of peptides S1–S5 on the HILIC column, run under the same conditions as in A and B. Peptides S2–S5 (all +2 net charge) were eluted from the column earlier than peptide S1 (+3), as would be expected with a cation-exchange separation. However, it is apparent that peptides S2–S5 were also resolved by a hydrophilic interaction mechanism, as the four peptides were eluted in order of increasing hydrophilicity [S5, the least hydrophilic (ΣR_c = 29.3) to S2, the most hydrophilic (ΣR_c = 17.5) (Table I)]. This result indicated that the dominant interactions between the peptides and the HILIC sorbent are ionic in character and that hydrophilic interactions are then additive to provide the mixed-mode separation.

Although Figs. 1 and 2 demonstrated the importance of ionic interactions during separations of basic peptides, hydrophilic interactions were also certainly a major factor influencing their retention behavior. We now wished to determine whether the column could retain peptides with no charge, *i.e.*, whether the HILIC column could retain and separate peptides *via* hydrophilic interactions only. The model peptides selected to clarify this point were the anion-exchange standards A1–A4 (containing one to four acidic residues, respectively; Table I). As stated previously, at pH 2, these peptides are uncharged, as their side-chain carboxyl groups are protonated. Fig. 3A, shows the reversed-phase separation of the four peptides on the C_{18} column. The order of peptide elution is based on increasing peptide hydrophobicity (Table I), with the most hydrophilic peptide, A1 (ΣR_c = 17.8), being eluted first, followed by A2

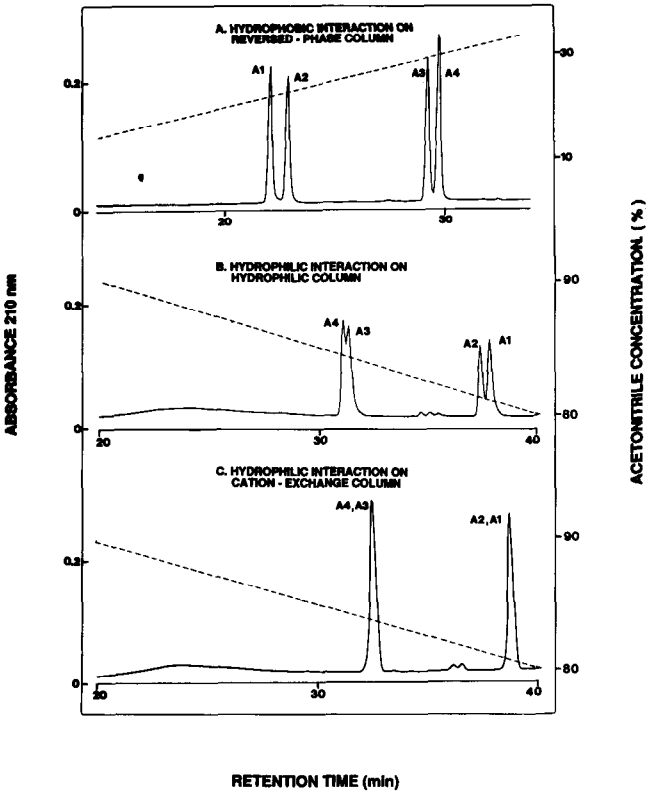

Fig. 3. Separation of a mixture of synthetic neutral peptides by (A) RP-HPLC, (B) HILIC on a HILIC column and (C) HILIC on a strong cation-exchange column. (A) Column, SynChropak RP-P C_{18} (250 × 4.6 mm I.D.); mobile phase, linear A–B increasing acetonitrile gradient (1% B/min) at a flow-rate of 1 ml/min, where A is 0.05% aqueous TFA and B is 0.05% TFA in acetonitrile. (B) Column, Polyhydroxyethylaspartamide HILIC column (200 × 4.6 mm I.D.); mobile phase, linear A–B decreasing acetonitrile gradient (0.5% B/min, following 10-min isocratic elution with 95% A–5% B), where A is 0.2% orthophosphoric acid in acetonitrile and B is 0.2% aqueous orthophosphoric acid. (C) Column, Polysulfoethylaspartamide strong cation-exchange column (200 × 4.6 mm I.D.); mobile phase as in B; temperature, 26°C. The dotted lines denote the respective acetonitrile gradients. The sequences of peptides A1, A2, A3 and A4 are shown in Table I.

(ΣR_c = 17.9), A3 (ΣR_c = 24.8) and finally A4 (ΣR_c = 24.9), the most hydrophobic peptide. In contrast, when HILIC was carried out with these peptides on the HILIC column (Fig. 3B), their retention and separation were clearly based on hydrophilic interactions with a reversal in elution order to that obtained by RP-HPLC (Fig. 3A), *i.e.*, the peptides were now eluted in order of increasing hydrophilicity. The resolution of the four peptides was not as effective on the HILIC column (Fig. 3B) compared with the reversed-phase column (Fig. 3A), even though the decreasing gradient slope of the HILIC run was shallower (0.5% acetonitrile/min) than that of the increasing gradient slope of the RP-HPLC run (1% B/min). However, the HILIC column was still able to resolve partially the two peptide pairs A1–A2 and A3–A4.

It seemed reasonable to assume that, by its nature, the charged character of a cation-exchange sorbent would also possess considerable hydrophilic character. Fig. 3C shows the HILIC separation of peptides A1–A4 on the polysulfoethylaspartamide strong cation-exchange column run under the same conditions as the HILIC column (Fig. 3B). Even though the cation-exchange column could not resolve the two peptide pairs A1–A2 and A3–A4, the column clearly retained the peptides based on hydrophilic interactions, with the least hydrophilic peptide pair, A3–A4, being eluted first and the most hydrophilic pair, A1–A2, being eluted last.

The polysulfoethylaspartamide cation exchanger is an hydrophilic silica-based packing. The Mono S strong cation-exchange column, which contains an organic polyether-based sorbent, was demonstrated to exhibit some hydrophobic character that could be suppressed by the addition of acetonitrile to the mobile phase buffers [3]. Interestingly, Fig. 4A shows that, under conditions where the starting buffer does not contain acetonitrile, and with a combined increasing salt (5 m*M* KCl/min) and acetonitrile (1% B/min) gradient, peptides S1, S2 and S5 were separated by a mixed-mode mechanism. Thus, peptides S2 and S5 (+2 net charge) were well separated from S1 (+3 net charge) mainly by an ionic mechanism, while S5 was resolved from S2 by an *hydrophobic* interaction mechanism. Thus, S5, which is more hydrophobic than S2 (ΣR_c = 29.3 and 17.5, respectively, at neutral pH), was eluted later than the latter peptide. In contrast, under the same run conditions, the polysulfoethylaspartamide column exhibited a mixed ionic and *hydrophilic* separation mechanism (Fig. 4D). Peptide S1 (+3 net charge) was well resolved from peptides S2 and S5 (+2 net charge) mainly by ionic interactions with the column, whilst peptides S2 and S5 were separated through hydrophilic interactions (S2 is eluted later than the less hydrophilic S5). Thus, the separation of S2 and S5 is reversed when comparing the two cation-exchange columns (Fig. 4A and D). This reversal can be readily explained by the difference in hydrophobicity of the two sorbents [*i.e.*, the Mono S sorbent is more hydrophobic (less hydrophilic) than the hydrophilic polysulfoethylaspartamide sorbent].

Fig. 4B demonstrates that hydrophobic interactions between the Mono S column and the peptides can be overcome by the addition of 10% acetonitrile to buffers A and B; ideal cation-exchange chromatography was now observed and no separation of peptides with identical net positive charge was achieved (*i.e.*, S2, S3, S4 and S5, with net charges of +2, were not resolved). In contrast, identical run conditions on the polysulfoethylaspartamide column separated peptides S2–S5 by hydrophilic interaction (Fig. 4E). Acetonitrile does not overcome hydrophilic interactions; on the contrary, it promotes these interactions.

Fig. 4C demonstrates that the Mono S column could separate peptides by a mixed ionic and hydrophilic interaction mechanism by simply increasing the concentration of acetonitrile in the mobile phase to 50% (v/v). Thus, peptides S2–S5 (+2 net charge) were now separated by hydrophilic interactions. Under the same run conditions on the polysulfoethylaspartamide column (Fig. 4F), a similar, albeit improved, separation of peptides S2–S5 was observed. In fact, S2 and S3 (which differ only by a single methyl group; Table I) were not fully resolved on the Mono S column (Fig. 4C); in contrast, they showed baseline resolution on the polysulfoethylaspartamide column (Fig. 4F).

Although the dominating interactions exhibited by both cation-exchange col-

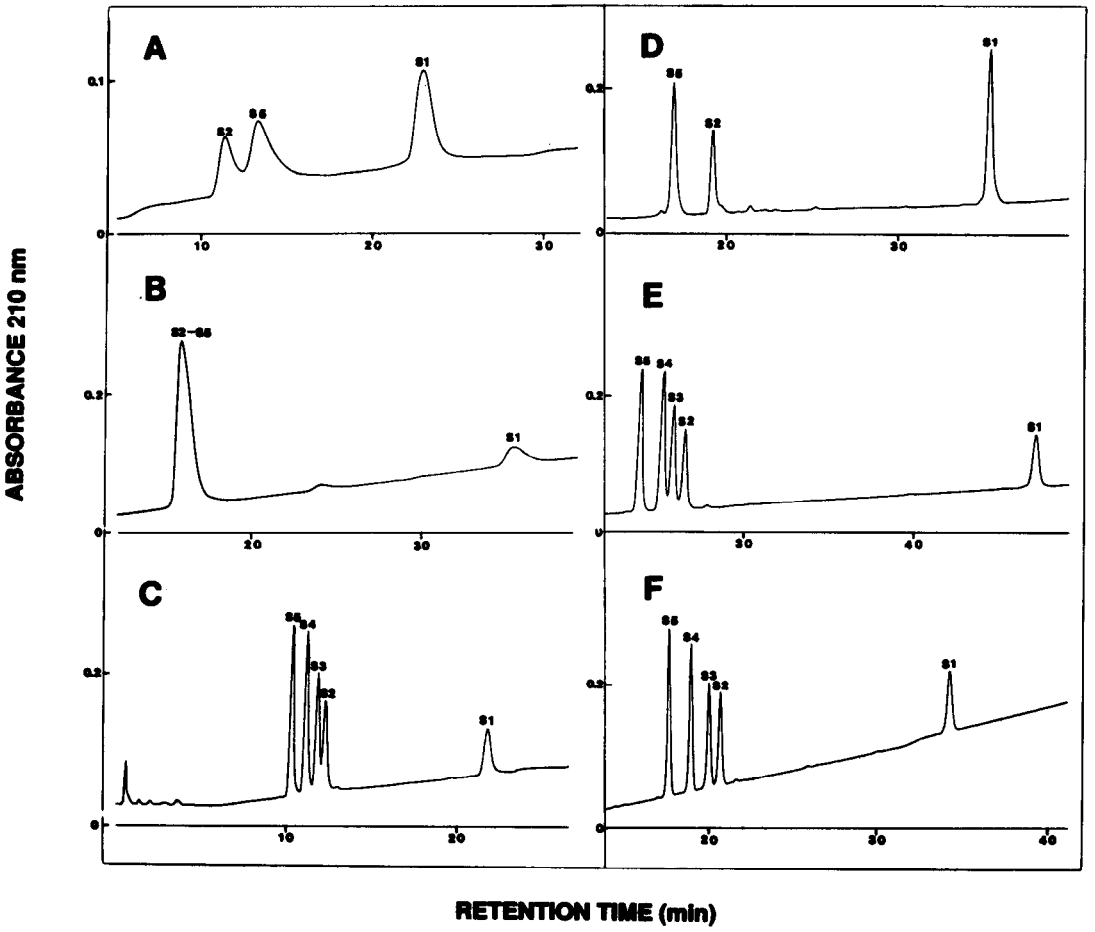

Fig. 4. Comparison of the mixed-mode separation of a mixture of synthetic positively charged peptides on two strong cation-exchange columns. Column: (A, B and C) non-silica-based Mono S HR 5/5 strong cation-exchange column (50 × 5 mm I.D.); (D, E and F) silica-based polysulfoethylaspartamide strong cation-exchange column (200 × 4.6 mm I.D.). Mobile phase: (A and D) linear A–B increasing salt and acetonitrile gradient (2% B/min, equivalent to 5 m*M* KCl/min and 1% acetonitrile/min) at a flow-rate of 1 ml/min, where buffer A is 5 m*M* aqueous KH_2PO_4 (pH 6.5) containing 50 m*M* KCl and buffer B is 5 m*M* aqueous KH_2PO_4 (pH 6.5) containing 0.25 *M* KCl and 50% (v/v) acetonitrile; (B and E) linear A–B increasing salt gradient (2% B/min, equivalent to 5 m*M* KCl/min) at a flow-rate of 1 ml/min, where buffer A is 5 m*M* aqueous KH_2PO_4 (pH 6.5) and buffer B is buffer A plus 0.25 *M* KCl, both buffers containing 10% (v/v) acetonitrile; (C and F) linear A–B increasing salt gradient (2% B/min, equivalent to 5 m*M* KCl/min) at a flow-rate of 1 ml/min, where buffer A is 5 m*M* aqueous KH_2PO_4 (pH 6.5) and buffer B is buffer A plus 0.25 *M* KCl, both buffers containing 50% (v/v) acetonitrile. Temperature, 26°C. The sequences of peptides S1–S5 are shown in Table I.

umns were ionic, with hydrophilic and hydrophobic interactions secondary, the performance of the polysulfoethylaspartamide column was always superior under the different sets of run conditions employed in Fig. 4. This is evidenced, for example, by the improved separation and peak shape of peptides S2–S5 on this column (Fig. 4D, E and F) compared with the Mono S column (Fig. 4A, B and C) under the same run

conditions. In addition, a good separation of these peptides on the polysulfoethylaspartamide column compared with the Mono S column was achieved with a lower level of acetonitrile in the mobile phase buffers: compare Fig. 4E (where only 10% of acetonitrile was required to produce the illustrated elution profile on the polysulfoethylaspartamide column) with Fig. 4C, where 50% of acetonitrile was required to achieve a similar separation on the Mono S. Low acetonitrile concentrations are advantageous for most peptides, which are more soluble in highly aqueous media. The column capacity of the former column was also considerably greater than that of the Mono S column. Thus, the retention times of the five peptides were considerably longer on the polysulfoethylaspartamide column (Fig. 4F) than on the Mono S column (panel Fig. 4C) under identical run conditions. This increased capacity of ionic groups on the polysulfoethylaspartamide column could also contribute to the improved hydrophilic interaction chromatography observed on this column during mixed-mode operation (Fig. 4). If one must operate a column in a mixed mode, it is preferable to have only two types of interactions, rather than three as observed with the Mono S column (Fig. 4). The hydrophobic interactions on the Mono S column are not advantageous in that they cause peak broadening and must be overcome just to obtain elution of more highly charged peptides [3].

CONCLUSIONS

Even though the ideal hydrophilic interaction sorbent has yet to be developed, it is certainly worthwhile continuing the development of such a sorbent lacking ionic characteristics. At present, the hydrophilic sorbent of the polysulfoethylaspartamide strong cation-exchange column utilized in this study is the most versatile. It provides excellent selectivity during operation in a mixed mode where conditions are selected to promote hydrophilic and ionic interactions, and this selectivity is achievable at a low acetonitrile concentration in the mobile phase. This cation-exchange column could also provide excellent selectivity changes with pH, which result in changes in the net charge on many peptides as the carboxyl groups are ionized with increasing pH. This combined effect of utilizing pH and hydrophilicity in cation-exchange chromatography may well rival reversed-phase chromatography for peptide applications. This mixed-mode separation in cation-exchange columns may also be superior in terms of selectivity to those achieved by capillary zone electrophoresis.

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REFERENCES

1 A. J. Alpert, *J. Chromatogr.*, 499 (1990) 177.
2 C. T. Mant and R. S. Hodges, *LC · GC*, 4 (1986) 250.
3 T. W. L. Burke, C. T. Mant, J. A. Black and R. S. Hodges, *J. Chromatogr.*, 476 (1989) 377.
4 D. Guo, C. T. Mant, A. K. Taneja, J. M. R. Parker and R. S. Hodges, *J. Chromatogr.*, 359 (1986) 499.

5 C. T. Mant and R. S. Hodges, in K. M. Gooding and F. E. Regnier (Editors), *HPLC of Biological Macromolecules: Methods and Applications*, Marcel Dekker, New York, 1990, pp. 301–332.
6 C. T. Mant, N. E. Zhou and R. S. Hodges, in E. Heftmann (Editor), *Chromatography, Part B*, Elsevier, Amsterdam, 5th ed., in press.
7 K. E. Bij, Cs. Horváth, W. R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
8 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 218 (1981) 497.
9 D. W. Armstrong and R. E. Boehm, *J. Chromatogr. Sci.*, 22 (1984) 379.
10 R. S. Blanquet, K. H. Bui and D. W. Armstrong, *J. Liq. Chromatogr.*, 9 (1986) 1933.
11 R. J. Simpson, R. L. Moritz, E. Nice and B. Grego, *Eur. J. Biochem.*, 165 (1987) 21.
12 R. J. Simpson and R. L. Moritz, in C. T. Mant and R. S. Hodges (Editors), *HPLC of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, 1991, pp. 399–408.
13 D. Guo, C. T. Mant and R. S. Hodges, *J. Chromatogr.*, 386 (1987) 205.
14 D. L. Crimmins, J. Gorka, R. S. Thoma and B. D. Schwartz, *J. Chromatogr.*, 443 (1988) 63.
15 A. J. Alpert and P. C. Andrews, *J. Chromatogr.*, 443 (1988) 85.