Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1009 (2003) 61–71
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Comparison of reversed-phase liquid chromatography and hydrophilic interaction/cation-exchange chromatography for the separation of amphipathic α-helical peptides with L- and D-amino acid substitutions in the hydrophilic face

Eva Hartmann[a,c], Yuxin Chen[b], Colin T. Mant[c], Alois Jungbauer[a], Robert S. Hodges[a,c,*]

[a]*Institute of Applied Microbiology, University of Agricultural Sciences, 1190 Vienna, Austria*
[b]*Department of Biochemistry, University of Alberta, Edmonton T6G 2H7, Canada*
[c]*Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, CO 80262, USA*

Abstract

Mixed-mode hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) is a novel high-performance technique which has excellent potential for peptide separations. Separations by HILIX/CEX are carried out by subjecting peptides to linear increasing salt gradients in the presence of high levels of acetonitrile, which promotes hydrophilic interactions overlaid on ionic interactions with the cation-exchange matrix. In the present study, HILIC/CEX has been compared to reversed-phase liquid chromatography (RP-HPLC) for separation of mixtures of diastereomeric amphipathic α-helical peptide analogues, where L- and D-amino acid substitutions were made in the centre of the hydrophilic face of the amphipathic α-helix. Unlike RP-HPLC, temperature had a substantial effect on HILIC/CEX of the peptides, with a rise in temperature from 25 to 65 °C increasing the retention times of the peptides as well as improving resolution. Our results again highlight the potential of HILIC/CEX as a peptide separation mode in its own right as well as an excellent complement to RP-HPLC.

Keywords: Mixed-mode chromatography; Hydrophilic interaction chromatography; Peptides

1. Introduction

Although reversed-phase liquid chromatography (RP-HPLC) is generally the method of choice for separation of peptide mixtures [1,2], this laboratory has previously shown mixed-mode hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) to be an excellent complement to RP-HPLC [3–10]. Indeed, HILIC/CEX, which combines the most advantageous aspects of two widely different separation mechanisms, i.e., a separation based on hydrophilicity/hydrophobicity differences between peptides overlaid on a separation based on net charge, has rivalled or even exceeded RP-HPLC for specific peptide mixtures [4,6,9]. For example, for the separation of two synthetic peptides and their deletion impurities (serine and cysteine), HILIC/CEX was clearly superior to RP-HPLC [6]. In another study [9], a two-step protocol consisting of HILIC/CEX followed by RP-HPLC was required for

*Corresponding author. Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, CO 80262, USA. Tel.: +1-303-315-8837; fax: +1-303-315-1153.
E-mail address: robert.hodges@uchsc.edu (R.S. Hodges).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00620-4

the successful purification of a 21-residue synthetic amphipathic α-helical peptide from serine side-chain acetylated impurities, with HILIC/CEX proving to be highly sensitive to subtle differences in hydrophilicities between the acetylated peptides and the desired product. Mixed-mode HILIC/CEX has also been used for the separation of H1 histones [11,12], proteins notoriously difficult to separate by traditional high-performance liquid chromatography (HPLC) techniques.

In an earlier study [7], we examined the potential of both RP-HPLC and HILIC/CEX for the separation of amphipathic α-helical peptides. Such peptides were represented by model α-helical peptides varying in amphipathicity and the nature of the side-chain substituted in the centre of the hydrophilic or hydrophobic face of the helix. Aside from clarifying the relative values of HILIC/CEX and RP-HPLC for specific model peptide separations, our results had wider implications for resolving complex peptide mixtures such as those characteristic of protein digests, where the occurrence of peptides with amphipathic α-helices is commonplace. In the present study, we now wished to examine further the relative effectiveness of RP-HPLC and HILIC/CEX in separating amphipathic α-helical peptides. Specifically, we looked to ascertain the ability of these techniques to separate mixtures of diastereomeric peptide analogues of a biologically active amphipathic α-helix denoted native (V_{681}) [13,14]. Thus, diastereomeric peptide pairs were prepared with either an L-amino acid or its D-amino acid enantiomer in the centre of the polar face of the amphipathic α-helix. Thus, each diastereomeric peptide pair has the same inherent hydrophilicity/hydrophobicity but potentially different amphipathicity due to the helix-disrupting properties of D-amino acids when substituted into an α-helix made up entirely of L-amino acids [15–19], providing a potent test for the resolving power of RP-HPLC and HILIC/CEX.

2. Experimental

2.1. Materials

HPLC-grade water was prepared by an E-pure water purification system from Barnstead International (Dubuque, IA, USA). Trifluoroacetic acid (TFA) was obtained from Halocarbon Products (River Edge, NJ, USA). Acetonitrile was obtained from EM Science (Gibbstown, NJ, USA). ACS-grade orthophosphoric acid and triethylamine (TEA) were obtained from Anachemia (Toronto, Canada). Sodium perchlorate was obtained from BDH (Poole, UK).

2.2. Columns

Analytical RP-HPLC runs were carried out on a Zorbax SB-300-C_8 column (150×2.1 mm I.D., 5 μm particle size; 300 Å pore size) from Agilent Technologies (Little Falls, DE, USA). Mixed-mode HILIC/CEX runs were carried out on a poly(2-sulfoethyl aspartamide)-silica (PolySulfoethyl A) strong cation-exchange column (200×2.1 mm I.D., 5 μm, 300 Å) from PolyLC (Columbia, MD, USA).

2.3. Instrumentation

Analytical RP-HPLC and HILIC/CEX runs were carried out on an Agilent 1100 Series liquid chromatograph. Peptide synthesis was carried out on an Applied Biosystems peptide synthesizer Model 430A (Foster City, CA, USA).

2.4. Peptide synthesis and purification

Synthesis of the peptides was carried out by standard solid-phase synthesis methodology using N^{α}-*tert*.-butyloxycarbonyl (t-Boc) chemistry on MBHA (methylbenzhydrylamine) resin (0.97 mmol/g) as described previously [19]. The crude peptides were purified by preparative RP-HPLC on an Applied Biosystems 400 solvent-delivery system connected to a 783A programmable absorbance detector. Amino acid analyses of purified peptides were carried out on a Beckman Model 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA, USA) and the correct primary ion molecular masses of peptides were confirmed by mass spectrometry on a Fisons VG Quattro electrospray mass spectrometer (Fisons, Pointe-Claire, Canada).

3. Results and discussion

3.1. RP-HPLC versus HILIC/CEX: general principles

Although the general principles of HILIC/CEX have been described in detail previously [7,10], a brief overview of this mixed-mode technique is useful for the present study. Thus, the term hydrophilic interaction chromatography was originally introduced to describe separations based on solute hydrophilicity [20]. Separation by HILIC, therefore, in a manner similar to normal-phase chromatography (to which it is related), depends on hydrophilic interactions between the solutes and a hydrophilic stationary phase, i.e., solutes are eluted in order of increasing hydrophilicity (decreasing hydrophobicity). This is, of course, in direct contrast to RP-HPLC, where solutes are eluted from a hydrophobic stationary phase in order of increasing hydrophobicity (decreasing hydrophilicity).

Characteristic of HILIC separations is the presence of a high organic modifier concentration to promote hydrophilic interactions between the solute and the hydrophilic stationary phase. Taking this concept a step further, this laboratory [3,4] demonstrated how to take advantage of the inherent hydrophilic character of ion-exchange, specifically strong cation-exchange (CEX) columns, by subjecting peptide mixtures to linear salt gradients in the presence of high levels of organic modifier. Separations based on hydrophilicity are thus superimposed on separations based on charge, i.e., the overall separation is effected by a mixture of chromatographic modes, namely mixed-mode HILIC/CEX. Such an approach takes simultaneous advantage of both the charged character of peptides as well as any hydrophilic/hydrophobic properties they possess.

3.2. Synthetic model peptides used in this study

Fig. 1 shows the sequences of the synthetic model peptides, based on the native V_{681} (denoted S_L), with L-Ser at position 11 of the sequence, i.e., in the hydrophilic face of the amphipathic α-helix. This position (denoted as X11 in Fig. 1) was chosen for the substitution site since it was in the very centre of the hydrophilic face of the amphipathic α-helix and is, therefore, surrounded by a very hydrophilic environment comprised of Thr and Ser residues (classified as containing uncharged, polar side-chains) and Lys and His residues (classified as basic, potentially positively charged side-chains). In contrast, the hydrophobic face is comprised solely of non-polar residues: Ala (containing a small, slightly hydrophobic side-chain), Val (containing a larger, moderately hydrophobic side-chain), Leu and Ile (both containing bulky, strongly hydrophobic side-chains), and Phe and Trp (both containing aromatic, hydrophobic side-chains). Overall, the sizes of the hydrophilic and hydrophobic faces of the helix are essentially identical, enabling a good comparison of the relative efficacies of RP-HPLC and HILIC/CEX to separate different peptide analogues. It should be noted that the non-polar face of the amphipathic α-helix represents a preferred binding domain for RP-HPLC, i.e., this face will bind preferentially to a reversed-phase hydrophobic stationary phase [21,22]; conversely, the hydrophilic face should represent a preferred binding domain for a hydrophilic stationary phase such as the strong cation-exchange matrix employed for HILIC/CEX in the present study. Evidence for such hydrophilic preferred binding domains has been described previously by our laboratory for both amphipathic α-helical peptides [7] as well as cyclic amphipathic β-sheet peptides [8].

For the present study, the L- and D-amino acids chosen for substitution at position 11 of the peptide sequence represented a range of side-chain properties. Thus, the three non-polar residues, Leu, Val and Ala, contain side-chains of increasing size and hydrophobicity: A_L, $A_D < V_L$, $V_D < L_L$, L_D; Ser (S_L, S_D) contains a small, polar (i.e., hydrophilic) side-chain; finally, Lys (K_L, K_D) contains a positively charged side-chain. The peptide analogue substituted with Gly at position 11 (G) represents the situation where no side-chain is present at the centre of the hydrophilic face of the helix.

The native sequence, V_{681}, is known to have a high potential to form an α-helix [13,14], as determined by circular dichroism spectroscopy. In addition, it has been shown that, even where helix-disrupting D-amino acids are substituted into α-helical peptides, high helicity may still be attained (generally comparable to their L-amino acid substituted analogues) in

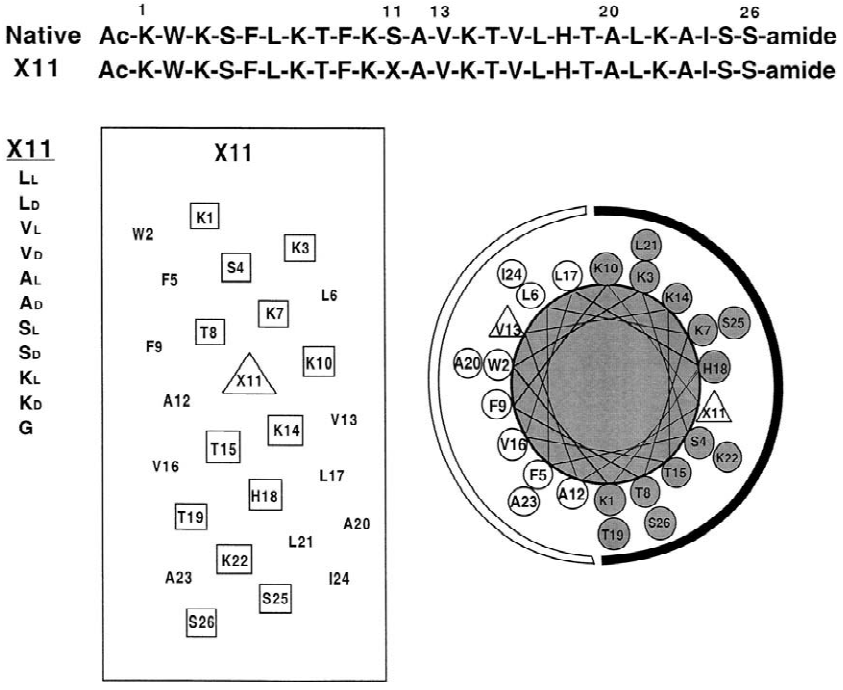

Fig. 1. Model synthetic amphipathic α-helical peptides. Top: Sequence of ''native'' peptide, denoted V_{681} (S_L), and sequence of peptide analogues, where X at position 11 is substituted by L-Leu (analogue denoted L_L, etc., for other L-analogues), D-Leu (analogue denoted L_D, etc., for other D-analogues), L-Val, D-Val, L-Ala, D-Ala, L-Ser (the native analogue V_{681}), D-Ser, L-Lys, D-Lys or G (denoted G). Bottom left: Helical net representation of the model peptide sequence, showing the hydrophilic face of the amphipathic α-helix; the substituted site at position 11 (X11) is highlighted by a triangle; the nomenclature of these analogues is listed to the left of the helical net. Bottom right: Helical wheel representation of the model peptide sequence; the substituted site at position 11 (X11) is highlighted by a triangle; residues in the hydrophilic face are shaded. The closed arc denotes the hydrophilic face; the open arc denotes the hydrophobic face. Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide.

the presence of helix-inducing solvents such as trifluoroethanol (TFE) [19]. Such was also the case in the present study, with high helicities for all L- and D-peptide analogues being obtained in 50% (v/v) TFE (data not reported). The run conditions characteristic of RP-HPLC (hydrophobic stationary phase, increasingly non-polar mobile phase) are well known to induce helical structure in potentially helical molecules [22–25]. Thus, the peptides used in the present study will be eluted as single-stranded amphipathic α-helices during RP-HPLC, interacting with the stationary phase through preferential binding with their hydrophobic faces. Further, it has also been previously shown that high concentrations of organic modifiers such as acetonitrile can induce helix formation in a potentially helical peptide [22,26]. Thus, under characteristic conditions of HILIC/CEX [high acetonitrile concentration in the mobile phase; 70% (v/v) in the present study], the peptide analogues would also be expected to be α-helical, allowing interaction of the hydrophilic face with the ion-exchange matrix [7].

3.3. RP-HPLC of amphipathic α-helical diastereomeric peptides

Fig. 2 shows the reversed-phase elution profiles of two mixtures of diastereomeric peptide pairs at 25 °C (top panels) and 65 °C (bottom panels). From Fig. 2, the D-substituted analogues were consistently eluted faster than their corresponding diastereomers. This decrease in retention time of the D-analogues compared to the L-analogues can be rationalized as being due to disruption of the amphipathic α-helix due to

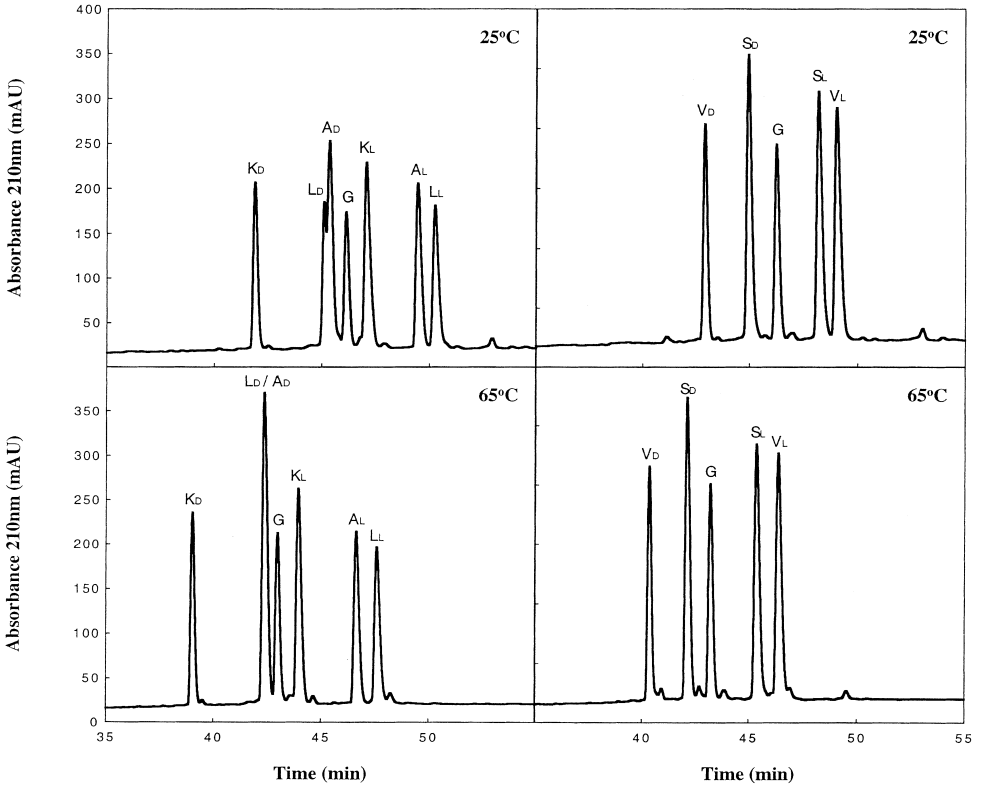

Fig. 2. RP-HPLC of diastereomeric amphipathic α-helical peptides. Column: reversed-phase Zorbax SB-300-C_8 (150×2.1 mm I.D.); see Experimental. Conditions: linear A–B gradient (1% B/min) at a flow-rate of 0.3 ml/min, where eluent A is 0.05% aq. TFA, pH 2.0, and eluent B is 0.05% TFA in acetonitrile. The sequences of the peptides are shown in Fig. 1.

the introduction of the D-amino acid [15–19], this disruption affecting both the hydrophobic face of the helix as well as the hydrophilic face where the substitution has been made. The overall effect on the non-polar face would be a decrease in the apparent hydrophobicity of this face when the helix is substituted with a D-amino acid relative to its L-diastereomer and, hence, a decrease in retention time of the former compared to the latter. Also from Fig. 2, the elution order of the analogues is generally in order of increasing hydrophobicity of the substituted residues within the L-analogues, i.e., $K_L < A_L < L_L$ (Fig. 2, left panels) and $S_L < V_L$ (Fig. 2, right panels). However, this is not necessarily true with the D-substituted analogues. Thus, A_D and L_D are almost co-eluted at 25 °C (Fig. 2, top left panel), with L_D being eluted just prior to A_D at 25 °C (top left panel); from Fig. 2, right panels, V_D, with a hydrophobic side-chain is eluted prior to S_D, which contains a polar, hydrophilic side-chain. This observation is likely due to the varying magnitude of disruption of the preferred non-polar binding domain of the peptide helix when different D-amino acids are substituted into the sequence, i.e., different D-amino acids disrupt the non-polar face to differing extents, resulting in the RP-HPLC elution orders shown for the D-analogues. Considering the elution of V_D significantly prior to S_D, for instance, reflects the observation by Chen et al. [19] that amino acids, such as Val, with β-branched side-chains showed the greatest reduction in apparent side-chain hydrophobicity due to D-amino acid substitutions into the centre of the non-polar face of an amphipathic α-helix.

The elution behaviour of K_L (Fig. 2, left panels) relative to analogue G is of note, since it has been clearly shown in model random coil peptides [27,28] that substitution of a Gly residue by a positively charged Lys residue leads to a significant decrease in

peptide retention time during RP-HPLC at pH 2.0. In contrast, from Fig. 2 (left panels) K_L is eluted after the G analogue. This observation is likely due to the L-Lys amino acid being in the centre of the hydrophilic face of the amphipathic α-helix, i.e., on the opposite side of the hydrophobic face which binds preferentially to the reversed-phase matrix. Clearly, the overall hydrophilicity of the Lys side-chain still has an effect on peptide retention behaviour (witness its elution prior to the A_L and L_L analogues containing non-polar groups at the substitution site); however, this effect is likely diminished compared to the situation where L-Lys was substituted into the centre of the non-polar face of the helix and was therefore able to interact to a greater extent with the hydrophobic stationary phase. In other words, substitutions, whether hydrophobic or hydrophilic, when made in the hydrophilic face of an amphipathic α-helix are not part of the preferred binding domain interacting with the hydrophobic matrix and have consequently lesser effects than if located in the centre of the hydrophobic face.

Concerning the effect of temperature on the RP-HPLC elution behaviour of the peptides, all peptides showed a decrease in retention time at 65 °C (bottom panels) compared to 25 °C (top panels), with no significant effect on resolution. Indeed, the major effect of temperature on resolution was a further deterioration of the poor separation of L_D and A_D seen at 25 °C (Fig. 2, top left panel) when the temperature was raised to 65 °C (Fig. 2, bottom left panel), where the two peptides are now co-eluted.

3.4. HILIC/CEX of amphipathic α-helical diastereomeric peptides

Fig. 3 shows the HILIC/CEX elution profiles of the two mixtures of diastereomeric peptides at 25 °C (top panels) and 65 °C (bottom panels). In a similar manner to their RP-HPLC retention behaviour (Fig. 2), the D-substituted analogues were again consistently eluted faster than their corresponding diastereomers (Fig. 3). As noted above for the RP-HPLC resolution of diastereomeric peptide pairs (Fig. 2), this separation by HILIC/CEX (Fig. 3) is probably a result of disruption of the preferred binding domain (in this case the hydrophilic preferred binding domain represented by the polar face of the amphipathic α-helix) by a D-amino acid substitution into the centre of the hydrophilic face of the helix. Interestingly, the observation that the D-analogues are eluted prior to their respective L-analogues suggests that the apparent hydrophilicity of the preferred binding domain is reduced by an L- to D-amino acid substitution in the centre of the polar face of the α-helix. Also from Fig. 3, the elution order of the analogues is in order of increasing hydrophilicity of the substituted residues within both the L- and D-analogues, i.e., L<A<K (Fig. 3, left panels) and V<S (Fig. 3, right panels).

The effect of temperature on the HILIC/CEX elution behaviour of the peptides (Fig. 3) is quite distinct to that of its effect during RP-HPLC (Fig. 2), with the retention times of the peptides all increasing on raising the temperature from 25 °C (top panels) to 65 °C (bottom panels). In addition, resolution of the peptides is greatly improved at the higher temperature. Thus, A_L and G are co-eluted at 25 °C (Fig. 3, top left) but mainly resolved at 65 °C (Fig. 3, bottom left); the L_L/L_D, A_L/A_D and K_L/K_D peptide pairs are also better separated at 65 °C (bottom left) compared to 25 °C (top left). From Fig. 3 (right panels), the improvement in separation of V_D and V_L at 65 °C (bottom right) compared to 25 °C (top right) is quite clear, as is the improvement in resolution of S_D, G and S_L.

3.5. Comparison of RP-HPLC and HILIC/CEX of amphipathic α-helical diastereomeric peptides at 25 and 65 °C

Table 1 summarizes retention time data for the L- and D-peptide analogues during RP-HPLC (Fig. 2) and HILIC/CEX (Fig. 3) at temperatures of 25 and 65 °C. From Table 1, the effect of raising the temperature of the separation from 25 to 65 °C has now been quantified by the expression Δt_R(65−25 °C), denoting the retention time change for each peptide with the temperature rise. As illustrated by the elution profiles shown in Fig. 2, all peptides show a decrease in RP-HPLC retention time (i.e., a negative Δt_R value) as the temperature is increased. In addition, this negative value is very similar for all peptides shown in Table 1: an average of $\Delta t_R = -2.8 \pm 0.4$ min. In contrast, and as illustrated in Fig. 3, all peptides show a quite significant increase in

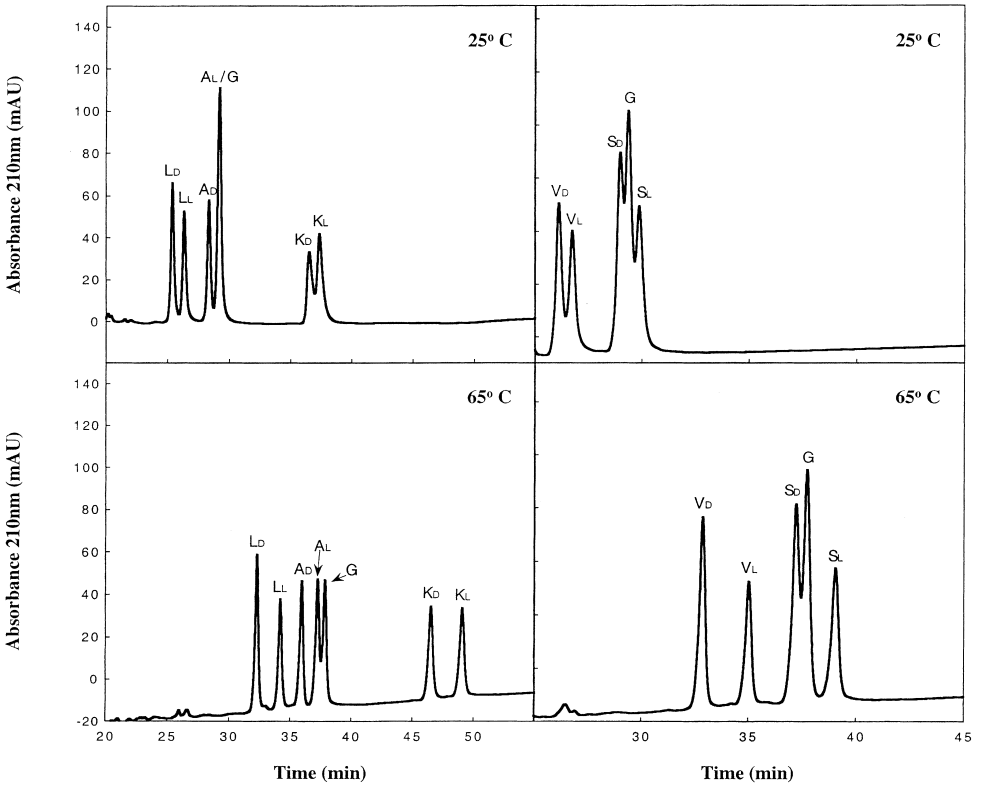

Fig. 3. HILIC/CEX of diastereomeric amphipathic α-helical peptides. Column: strong cation-exchange PolySulfoethyl A (200×2.1 mm I.D.); see Experimental. Conditions: linear A–B gradient (5 m*M* $NaClO_4$ to 250 m*M* $NaClO_4$ in 50 min) at a flow-rate of 0.3 ml/min, where eluent A is 5 m*M* aq. triethylammonium phosphate (TEAP), pH 4.5, containing 5 m*M* $NaClO_4$ and eluent B is 5 m*M* aq. TEAP, pH 4.5, containing 250 m*M* $NaClO_4$, both eluents also containing 70% (v/v) acetonitrile. The sequences of the peptides are shown in Fig. 1.

retention time with the temperature change from 25 to 65 °C. Points to note include the observation that the effect of the temperature rise is consistently greater for the L-substituted analogues compared to the D-substituted peptides. The Δt_R values for the L-analogues and D-analogues show an average of +8.4 and +7.4 min, respectively, excluding G, K_L and K_D. Interestingly, the effect of the rise in temperature from 25 to 65 °C appeared to have a greater effect on K_L and K_D (Δt_R values of 11.6 and 10.0 min, respectively) than the other peptide analogues, suggesting that the presence of an extra positive charge (which is concomitantly a significant increase in hydrophilicity of the polar face of the helix) enhances the effect of temperature during HILIC/CEX. Indeed, although subtle, the greater Δt_R values of S_L (+9.3 min) and S_D (+8.3 min) relative to the peptides substituted with non-polar Ala, Leu and Val residues (Δt_R ranges of +7.8 to +8.3 min for the L-analogues and +6.9 to +7.6 min for the D-analogues) support the conclusion that the effect of temperature during HILIC/CEX is related to the overall hydrophilicity of the polar face of the α-helix, i.e., the more hydrophilic (less hydrophobic) the preferred binding domain, the greater the increase in retention time with increasing temperature.

This observation is illustrated graphically in Fig. 4 which plots the effect of raising the temperature from 25 to 65 °C on the retention times of L_L (representing an amphipathic helix with a bulky, hydrophobic side-chain substituted into the centre of the hydrophilic face), S_L (substituted with a polar, uncharged side-chain), and K_L (substituted with a positively charged side-chain). Also included is the effect of temperature on a 10-residue random coil peptide standard, S5. From Fig. 4 (top), the small, and

Table 1
Effect of temperature on peptide retention behaviour in RP-HPLC and HILIC/CEX

Peptide[a]	RP-HPLC[c]			HILIC/CEX[b]		
	t_R 25 °C	t_R 65 °C	Δt_R (65−25 °C)	t_R 25 °C	t_R 65 °C	Δt_R[d] (65−25 °C)
Mix 1						
L_L	50.3	47.6	−2.7	26.4	34.2	+7.8
L_D	45.1	42.4	−2.7	25.4	32.3	+6.9
A_L	49.5	46.6	−2.9	29.2	37.3	+8.1
A_D	45.3	42.4	−2.9	28.4	36.0	+7.6
G	46.1	43.0	−3.1	29.2	37.9	+8.7
K_L	47.1	43.9	−3.2	37.4	49.0	+11.6
K_D	41.9	39.0	−2.9	36.5	46.5	+10.0
Mix 2						
V_L	49.0	46.4	−2.6	26.8	35.1	+8.3
V_D	42.9	40.4	−2.5	26.1	33.0	+6.9
S_L	48.1	45.4	−2.7	29.9	39.2	+9.3
S_D	44.9	42.1	−2.8	29.0	37.3	+8.3
G	46.2	43.2	−3.0	29.3	37.7	+8.4

[a] Sequence and denotion of peptides shown in Fig. 1.
[b] RP-HPLC conditions shown in Fig. 2. Column, reversed-phase Zorbax SB-300-C_8.
[c] HILIC/CEX conditions shown in Fig. 3. Column, strong cation-exchange (PolySulfoethyl A).
[d] Δt_R refers to the retention time of a peptide at 65 °C (t_R 65 °C) minus its retention time at 25 °C (t_R 25 °C).

similar, effect of temperature on RP-HPLC of the three α-helical peptides is quite clear, with essentially parallel decreasing profiles as the temperature is raised from 25 to 65 °C. Indeed, the profiles of these three peptides were also similar to that of the random coil peptide standard, S5, albeit the latter had a slightly less steep profile. In contrast, the profiles for the three helical peptides during HILIC/CEX (Fig. 4, bottom) were somewhat more distinct from each other, as well as from the random coil S5, as the temperature was raised from 25 to 65 °C. Thus, the positive slopes of the three helical peptides increased in the order $L_L<S_L<K_L$, i.e., in order of increasing hydrophilicity of the substituted side-chain at the centre of the hydrophilic face of the helix, as noted previously. Interestingly, the positive slope of S5 was considerably shallower than those of the three helical peptides, i.e., the retention time of this peptide with negligible secondary structure increased little with a rise in temperature compared to the amphipathic α-helical peptides. It is also interesting to note that despite the reversal in retention time order between the two HPLC modes ($K_L<S_L<L_L$ for RP-HPLC Fig. 4, top and $L_L<S_L<K_L$ for HILIC/CEX Fig. 4, bottom), the random coil peptide S5 was eluted first in both modes. This again suggests a link between the retention behaviour of peptides with a defined conformation (in this case α-helical peptides with defined hydrophilic and hydrophobic faces) during RP-HPLC and HILIC/CEX compared to a peptide with negligible secondary structure.

Also from Fig. 4, it is important to note that, under the conditions employed for RP-HPLC and HILIC/CEX in this study, the three L-analogues were considerably better separated by HILIC/CEX (Fig. 4, bottom) compared to RP-HPLC (Fig. 4, top). These results reflect previous results from this laboratory [7] that amphipathic α-helical peptides with substitutions made in the hydrophilic face of the helix are likely to be better separated by HILIC/CEX compared to RP-HPLC. A more thorough investigation of this phenomenon for the present peptide analogues (where L- and D-amino acids are substituted into the hydrophilic and hydrophobic faces of the amphipathic α-helices) is the subject of a separate study [29].

Table 2 now examines the effect of temperature on the separation of diastereomeric peptide pairs. From Table 2, there is no clear link between the inherent

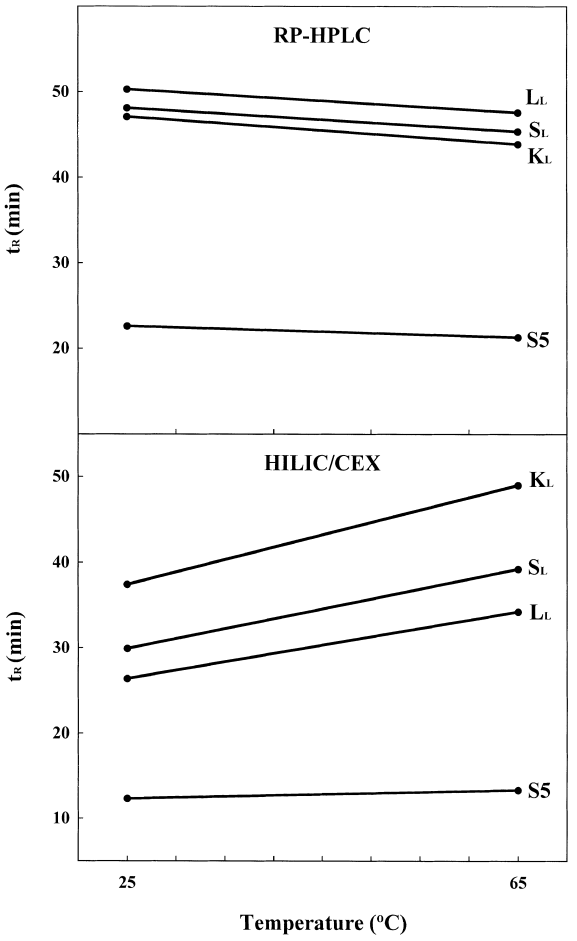

Fig. 4. Effect of temperature on retention behaviour of diastereomeric amphipathic α-helical peptides in RP-HPLC (top) and HILIC/CEX (bottom). Columns: (top panel) reversed-phase Zorbax SB-300-C_8 (150×2.1 mm I.D.) and (bottom panel) strong cation-exchange PolySulfoethyl A (200×2.1 mm I.D.); see Experimental. Conditions for HILIC/CEX and RP-HPLC as in Figs. 2 and 3, respectively. The sequences of the peptides are shown in Fig. 1. The sequence of the random coil peptide standard, S5, is Ac-Arg–Gly–Val–Val–Gly–Leu–Gly–Leu–Gly–Lys-amide; Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide.

hydrophobicity/hydrophilicity of a particular side-chain and the decrease in retention time in both HPLC modes (Δt_R 25 °C, Δt_R 65 °C) when the L-amino acid is substituted by its D-enantiomer. However, it can be seen that there is a smaller difference in Δt_R values between L- and D-peptide pairs during HILIC/CEX compared to RP-HPLC at both 25 and 65 °C. In contrast, the separation between such peptide pairs is enhanced during HILIC/CEX when raising the temperature ($\Delta\Delta t_R$ values ranging from +0.5 to +1.6 min) compared to the negligible effect during RP-HPLC ($\Delta\Delta t_R$ values ranging from −0.3 to +0.1 min, the negative values for V_L/V_D and K_L/K_D actually indicating a deterioration in the separation.

3.6. Comparison of selectivity of RP-HPLC and HILIC/CEX separation of amphipathic α-helical diastereomeric peptides

Fig. 5 compares the selectivity of RP-HPLC (top panels) and HILIC/CEX (bottom panels) for the separation of a mixture of seven amphipathic α-helical diastereomeric peptides at 25 °C (left panels) and 65 °C (right panels). As the arrows highlight for the 25 °C runs (Fig. 5, left panels), there is a considerable switch in elution order of the peptides between the two HPLC modes, underlining the useful complementary nature of RP-HPLC and HILIC/CEX. Of note here also is the clear illustration of the greater time required to elute the seven peptides during RP-HPLC (a range of 41.9 min for K_D to 50.2 min for L_L) compared to HILIC/CEX (25.4 min for L_D to 37.4 min for K_L) under the conditions employed for the two HPLC modes. However, this difference is strikingly diminished when the temperature is raised to 65 °C. Thus, from Fig. 5 (right panels), due to the small reduction in RP-HPLC retention times with the rise in temperature concomitant with the significant increase in HILIC/CEX retention times, the retention time range between the two modes is now more similar, allowing perhaps a more valid comparison of the complementary selectivity differences between RP-HPLC and HILIC/CEX of these peptides. In addition, the superior resolution of the peptides in this particular peptide mixture by HILIC/CEX at 65 °C compared to RP-HPLC at this temperature (and, indeed, to RP-HPLC at 25 °C) is well illustrated.

4. Conclusions

We have compared the ability of RP-HPLC and HILIC/CEX to separate mixtures of amphipathic α-helical diastereomeric peptides, with L- and D-amino acid substitutions made in the centre of the

Table 2
Effect of temperature on separation of diastereomeric peptide pairs by RP-HPLC and HILIC/CEX

Peptide pair[a]	RP-HPLC[b]			HILIC/CEX[c]		
	Δt_R 25 °C[d]	Δt_R 65 °C[d]	$\Delta\Delta t_R$[e]	Δt_R 25 °C[d]	Δt_R 65 °C[d]	$\Delta\Delta t_R^e$
L_L/L_D	5.2	5.2	0	1.0	1.9	0.9
V_L/V_D	6.1	6.0	−0.1	0.7	2.1	1.4
A_L/A_D	4.2	4.2	0	0.8	1.3	0.5
S_L/S_D	3.2	3.3	0.1	0.9	1.9	1.0
K_L/K_D	5.2	4.9	−0.3	0.9	2.5	1.6

[a] Sequence and denotion of peptides shown in Fig. 1.
[b] RP-HPLC conditions are shown in Fig. 2. Column, reversed-phase Zorbax SB-300-C_8.
[c] HILIC/CEX conditions are shown in Fig. 3. Column, strong cation-exchange (PolySulfoethyl A).
[d] Δt_Rrefers to the retention time of the first peptide (L-analogue) shown in each pair of peptides minus the retention time of the second (D-analogue) peptide.
[e] $\Delta\Delta t_R$ is the Δt_R 65 °C value minus the Δt_R 25 °C value.

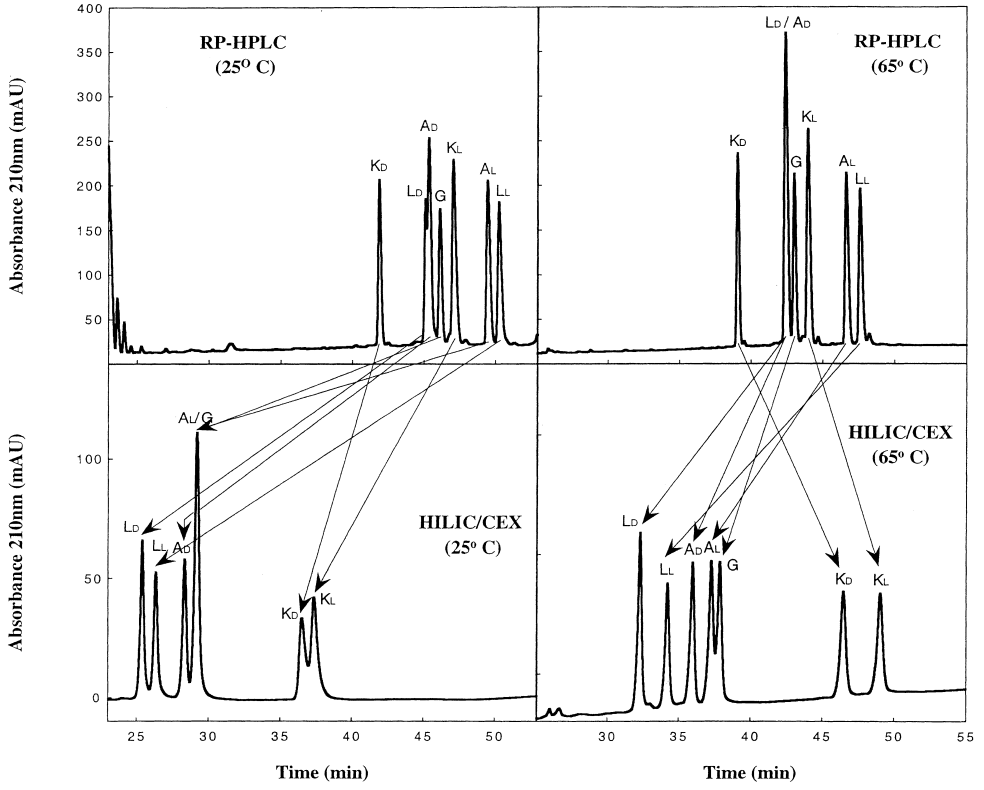

Fig. 5. Comparison of selectivity changes in the separation of diastereomeric amphipathic α-helical peptides by RP-HPLC (top) and HILIC/CEX (bottom) at 25 °C (left panels) and 65 °C (right panels). Columns: (top panels) reversed-phase Zorbax SB-300-C_8 (150×2.1 mm I.D.) and (bottom panels) strong cation-exchange PolySulfoethyl A (200×2.1 mm I.D.); see Experimental. Conditions for RP-HPLC and HILIC/CEX as in Figs. 2 and 3, respectively. The sequences of the peptides are shown in Fig. 1.

hydrophilic face of the α-helix. While both methods proved to be effective for separating such peptides, they did so often with greatly different selectivities, underlining the useful complementary nature of the two HPLC methods. Interestingly, temperature had a substantial effect on HILIC/CEX of the peptides, more so than RP-HPLC, with a rise in temperature from 25 to 65 °C both increasing the retention times of the peptides, as well as improving peptide separation. These results again stress the potential of HILIC/CEX, both as a peptide separation mode in its own right as well as an excellent complement to RP-HPLC.

Acknowledgements

This work was supported by an N.I.H. grant to R.S.H. (R01GM61855) and the Canadian Bacterial Diseases Network (R.S.H.). E.H. would like to thank co-author A.J. from the Institute of Applied Microbiology, University of Agricultural Sciences, Vienna, Austria, for making her exchange visit possible.

References

[1] C.T. Mant, R.S. Hodges (Eds.), HPLC of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991.
[2] C.T. Mant, R.S. Hodges, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, 2nd ed., Marcel Dekker, New York, 2002, p. 433.
[3] B.-Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 548 (1991) 13.
[4] B.-Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 594 (1992) 75.
[5] C.T. Mant, R.S. Hodges, Methods Enzymol. 271 (1996) 3.
[6] C.T. Mant, L.H. Kondejewski, P.J. Cachia, O.D. Monera, R.S. Hodges, Methods Enzymol. 289 (1997) 426.
[7] C.T. Mant, J.R. Litowski, R.S. Hodges, J. Chromatogr. A 816 (1998) 65.
[8] C.T. Mant, L.H. Kondejewski, R.S. Hodges, J. Chromatogr. A 816 (1998) 79.
[9] J.R. Litowski, P.D. Semchuk, C.T. Mant, R.S. Hodges, J. Peptide Res. 54 (1999) 1.
[10] C.T. Mant, R.S. Hodges, in: The Encyclopedia of Separation Science, Academic Press, 2000, p. 3615.
[11] H. Lindner, B. Sarg, C. Meraner, W. Helliger, J. Chromatogr. A 743 (1996) 137.
[12] H. Lindner, B. Sarg, C. Meraner, W. Helliger, J. Chromatogr. A 782 (1997) 55.
[13] L. Zhang, T. Falla, M. Wu, S. Fidai, J. Burian, W. Kay, R.E.W. Hancock, Biochem. Biophys. Res. Commun. 247 (1998) 674.
[14] L. Zhang, R. Benz, R.E.W. Hancock, Biochemistry 38 (1999) 8102.
[15] M.I. Aguilar, S. Mougos, J. Boublik, J. Rivier, M.T.W. Hearn, J. Chromatogr. 646 (1993) 53.
[16] S. Rothemund, M. Beyermann, E. Krause, G. Krause, M. Bienert, R.S. Hodges, B.D. Sykes, F.D. Sonnichsen, Biochemistry 34 (1995) 12954.
[17] S. Rothemund, E. Krause, M. Beyermann, M. Dathe, M. Bienert, R.S. Hodges, B.D. Sykes, F.D. Sonnichsen, Peptide Res. 9 (1996) 79.
[18] E. Krause, M. Bienert, P. Schmieder, H. Wenschuh, J. Am. Chem. Soc. 122 (2000) 4865.
[19] Y. Chen, C.T. Mant, R.S. Hodges, J. Peptide Res. 59 (2002) 18.
[20] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[21] V. Steiner, M. Schar, K.L. Bornsen, M. Mutter, Trends Biochem. Sci. 13 (1998) 360.
[22] N.E. Zhou, C.T. Mant, R.S. Hodges, Peptide Res. 3 (1990) 8.
[23] A.W. Purcell, M.I. Aguilar, R.E.W. Wettenhall, M.T.W. Hearn, Peptide Res. 8 (1995) 160.
[24] S.E. Blondelle, J.M. Ostresh, R.A. Houghten, E. Perez-Paya, Biophys. J. 68 (1995) 351.
[25] D.L. Steer, P.E. Thompson, S.E. Blondelle, R.A. Houghten, M.I. Aguilar, J. Peptide Res. 51 (1998) 401.
[26] S.Y.M. Lau, A.K. Taneja, R.S. Hodges, J. Chromatogr. 317 (1984) 129.
[27] D. Guo, C.T. Mant, A.K. Taneja, J.M.R. Parker, R.S. Hodges, J. Chromatogr. 359 (1986) 499.
[28] D. Guo, C.T. Mant, A.K. Taneja, R.S. Hodges, J. Chromatogr. 359 (1986) 519.
[29] Y. Chen, E. Hartmann, C.T. Mant, R.S. Hodges, in preparation.