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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

XCI[a]. THE INFLUENCE OF TEMPERATURE ON THE CHROMATOGRAPHIC BEHAVIOUR OF PEPTIDES RELATED TO HUMAN GROWTH HORMONE

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SUMMARY

The influence of temperature on the gradient elution properties of synthetic peptides related to residues [6–13] of human growth hormone, *e.g.*, Leu[1]-Ser-Arg-Leu-Phe-Asp-Asn-Ala[8], has been studied by using both an octadecylsilica and a polymeric fluorocarbon stationary phase. Correlation of changes in the solute hydrophobic contact area and affinity for the stationary phase, as given by S and $\log k_0$ values respectively, revealed that the α- and imide forms are more conformationally stable than the β-linked peptide. In addition, negative values of the standard entropy change, ΔS^0_{assoc}, for the transfer of the solute to the stationary phase, were observed for both α- and β-linked peptides. These results are indicative of an increased ordering of the system upon solute adsorption and implies that the open-chain peptides exist in solution in more flexible conformations, while the helical structure of the cyclised imide is more rigid and constrained. The implications of the relative conformational stability of these peptides in their role as insulin-potentiating agents is also discussed.

INTRODUCTION

The exquisite nature of high-performance liquid chromatographic techniques as physicochemical tools in the analysis of peptide and protein surface interactions is rapidly gaining wide recognition. The ability of the stationary phase to act as a probe of the topographic surface of the peptide or protein solute has provided greater insight into the factors that control the mechanistic basis of chromatographic separations[1–3]. In a previous study[1], we reported the analysis of the retention behaviour of a number of peptide analogues related to the amino-terminus of human growth hormone (hGH), separated under reversed-phase high-performance liquid chromatography (RP-HPLC) conditions. It was found that these peptides, which are derived from a helical segment of the parent protein, also exhibited chromatographic properties that were

[a] For Part XC, see ref. 1.

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consistent with the formation of a helical structure at the stationary phase interface. Furthermore, comparison of solute hydrophobic contact area and affinity for the stationary phase, as expressed by S and log k_0 values, at different mobile phase pH values has revealed specific details of the factors that control the orientation of the peptides at the hydrophobic stationary phase. The differences in the biological activity of the Asp^6 imide and the open-chain Asp^6 α- and β-forms of the parent sequence Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala is clearly related to the difference in molecular charge distribution and peptide conformation, both in solution and at the site of biological action. As part of our studies on defining the conformational preferences of these peptides, the relative stability of the imide and α- and β-forms of these peptides was investigated through the analysis of the influence of temperature on chromatographic properties.

MATERIALS AND METHODS

Apparatus

All chromatographic measurements were performed with a Du Pont 8800 liquid chromatograph (Du Pont, Wilmington, DE, U.S.A.) coupled to a Valco HPLC 6-port injector (Valco, Houston, TX, U.S.A.) and a Waters M450 variable-wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.). All measurements were routinely monitored at 215 nm and recorded with the aid of a Spectra-Physics SP4100 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.). Chromatographic measurements at 5 and 25°C were made by immersing the column in a plastic column jacket, coupled to a recirculating cooler (FTS Systems, New York, U.S.A.), while a DuPont column oven was used for measurements at 50, 65 and 75°C. RP-HPLC was carried out with a Bakerbond widepore octadecylsilica (J. T. Baker, Phillipsburg, NJ, U.S.A.) with a nominal particle diameter of 5 μm and average pore size of 30 nm, packed into a 25 cm × 0.46 cm I.D. column, and a Du Pont Bio Series Poly F proprietary polymeric fluorocarbon HPLC packing with a 20-μm particle size and 30-nm pore size, packed into a 8 cm × 0.62 cm I.D. column. All injections were made with SGE (Melbourne, Australia) syringes, and pH measurements were performed with an Orion Model SA520 pH meter (Orion, Cambridge, MA, U.S.A.).

Chemicals and reagents

Acetonitrile (HPLC grade) was obtained from Mallinckrodt (Paris, KY, U.S.A.), and trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.). Water was distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, U.S.A.). N-Acetyl-L-tryptophanamide and N-acetyl-L-phenylalanine methyl ester and penta-D-phenylalanine were all obtained from Sigma (St. Louis, MO, U.S.A.). The hGH-related peptide analogues were synthesised by established 9-fluorenylmethyloxycarbonyl (F-moc) group protection procedures and purified by RP-HPLC.

Chromatographic procedures

Bulk solvents and mobile phases were filtered and degassed under vacuum. Linear gradient elution was carried out with 0.1% TFA in water (buffer A) and 0.1% TFA in 50% aqueous acetonitrile (buffer B) over gradient times varying between 15

and 90 min and at a flow-rate of 1 ml/min. Peptide solutions were prepared by dissolving the peptide in 0.1% TFA at a concentration of 1 mg/ml, and sample sizes varied between 5 and 20 μg. All data points were derived from duplicate measurements with retention times typically varying by less then 1%. The column dead-time was taken as the retention time for sodium nitrate. The various chromatographic parameters used in the analysis of peptide retention and bandwidth behaviour were calculated using the Pek-n-ese program, written in BASIC language for a Hewlett-Packard HP86B computer, as previously described[2].

RESULTS AND DISCUSSION

Solute retention in adsorptive modes of HPLC is governed by the distribution equilibria established between the stationary phase and the mobile phase, and can be defined as

$$K = \frac{[P]_s}{[P]_m} \tag{1}$$

where $[P]_s$ and $[P]_m$ represent the solute concentration in the stationary phase and mobile phase, respectively.

The dependence of the median capacity factor, $\bar{k}$, of a solute, chromatographed under regular reversed phase conditions, on K, is then given by

$$\bar{k} = \phi K \tag{2}$$

where ϕ is equal to the ratio of the volume of the stationary phase, V_s, to the volume of the mobile phase, V_m.

The thermodynamic equilibrium constants can be equated with the overall standard unitary free-energy changes associated with the transfer of the solutes from the mobile phase to the stationary phase, such that

$$\log K = -\Delta G^0_{assoc}/RT \tag{3}$$

where R is the gas constant and T is the temperature. Solute retention can then be expressed as

$$\log \bar{k} = -\Delta G^0_{assoc}/RT + \log \phi \tag{4}$$

Thus, the dependence of $\bar{k}$ on T is given by

$$\log \bar{k} = \frac{-\Delta H^0_{assoc}}{RT} + \frac{\Delta S^0_{assoc}}{R} + \log \phi \tag{5}$$

where ΔH^0_{assoc} and ΔS^0_{assoc} are the standard enthalpy and entropy changes, respectively, for the transfer of the solute to the stationary phase.

The capacity factors of peptides in RP-HPLC are generally reduced with increased temperature. For peptides with no significant secondary or tertiary structure, higher temperatures will favour more rapid mass transfer of the solute and

TABLE I

PEPTIDE SEQUENCES AND S AND LOG k_0 VALUES OBTAINED AT DIFFERENT TEMPERATURES ON THE C_{18} COLUMN

Abbreviations for amino acids were as follows: A = Ala = alanine, D = Asp = aspartic acid, E = Glu = glutamic acid, F = Phe = phenylalanine, G = Gly = glycine, L = Leu = leucine, R = Arg = arginine, S = Ser = serine, W = Trp = tryptophan.

Peptide	*5°C*		*25°C*		*50°C*		*65°C*		*75°C*	
	S	$\log k_0$	S	$\log k_0$	S	$\log k_0$	S	$\log k_0$	S	$\log k_0$
1 LSRLFDNA (imide)	8.8	4.2	7.2	3.6	7.0	3.4	8.2	3.8	8.5	3.8
2 LFDNAG (α)	10.6	1.8	11.2	1.8	11.3	1.5	10.9	1.3	11.5	1.3
3 LSRLFENAG (β)	11.7	1.9	12.8	1.9	11.6	1.5	11.9	1.4	15.5	1.6
4 $(Phe)_5$	8.7	3.6	9.6	4.0	8.0	3.3	12.7	4.6	19.5	6.6
5 F	6.9	2.1	6.8	2.0	5.4	1.6	6.2	1.7	9.0	1.9
6 W	7.8	1.4	9.5	1.4	7.9	1.0	9.6	0.9	–	–

lead to improved efficiencies. Recently, the influence of temperature on a first-order irreversible on-column reaction was investigated[4]. These studies revealed that, depending on the rate constant and activation energy for the reaction, the deleterious effects of, *e.g.*, protein denaturation are diminished due to the faster separation times and decreased exposure of the solute to the high temperatures.

The influence of temperature on the chromatographic behaviour of peptides and proteins can also be used to characterise conformational equilibria and stability of the solutes. The correlation of changes in the hydrophobic contact area and solute affinity, as given by the S and $\log k_0$ values of the solute, can provide valuable insight into structural and environmental factors that influence chromatographic behaviour of peptides and proteins. The gradient elution behaviour of a set of peptides related to residues [6–13] of hGH has been recently studied. It was found that the closed-ring imide form and the open-chain α- and β-forms all chromatographed as an amphipathic helical structure with slight differences in the final conformation. The main factors contributing to the stabilisation of the amphipathic helical structure of the imide have been shown by 2D-NMR (NOESY) studies[5] to consist of the hydrophobic face of the helix and a dipolar interaction between an arginine residue (Arg^3) and the imide ring, in the open-chain forms. The influence of temperature on the reversed-phase gradient-elution properties of these peptides was therefore studied. Table I lists the peptides used in the present study. Gradient-elution data were accumulated at gradient times (t_G) of 15, 30, 45, 60, and 90 min and a flow-rate of 1 ml/min at temperatures of 5, 25, 50, 65, and 75°C. Samples were chromatographed, in the first instance, on a C_{18} stationary phase with a linear gradient from 0.1% TFA in water to 0.1% TFA in 50% aqueous acetonitrile. Plots of the median capacity factor, $\bar{k}$, *versus* the corresponding organic mole fraction, $\bar{\psi}$, were derived as described previously[2]. The S and $\log k_0$ values for each temperature were obtained by linear regression analysis of the plots according to the relationship

$$\log \bar{k} = \log k_0 - S\bar{\psi} \qquad (6)$$

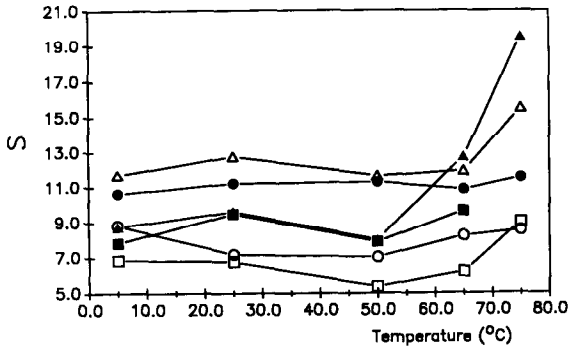

Fig. 1. Plot of *S versus* temperature (°C) for the peptides and amino acids listed in Table I. Experimental data points were derived from gradient retention data with t_G = 15, 30, 45, 60 and 90 min and a flow-rate of 1 ml/min, on a C_{18} column. See Materials and Methods section for other details. Peptides: (○), **1**; (●), **2**; (△), **3**; (▲), **4**; (□), **5**; (■), **6**.

The three hGH-related peptides included in this study represent examples of an imide, and an α- and β-linked peptide. In addition pentaphenylalanine [$(Phe)_5$] was used as a control as this peptide is known to exist as a helix in solution[6]. Data for the amino acid derivatives N-acetyl-L-tryptophanamide and N-acetyl-L-phenylalanine methyl ester were also determined as an example of non-interconverting solutes. The *S* and log k_0 values are listed in Table I and plotted against temperature in Figs. 1 and 2, respectively. Studies on the influence of temperature on the physical state of silica surfaces covered by alkylsiloxy layers have demonstrated[7] the existence of phase transitions which are characterised by an increase in the retention of molecular probes with increasing temperature. This conformational transition of the ligand is dependent on the carbon chain length, the ligand density and the nature of the mobile phase. While temperature-dependent stationary phase transitions may also occur with the octadecylsilica packing used in the present study, differences in the chromatographic properties of the peptide solutes will be related to the differences in amino acid composition and sequence. Further, the trends evident in these studies are expected to

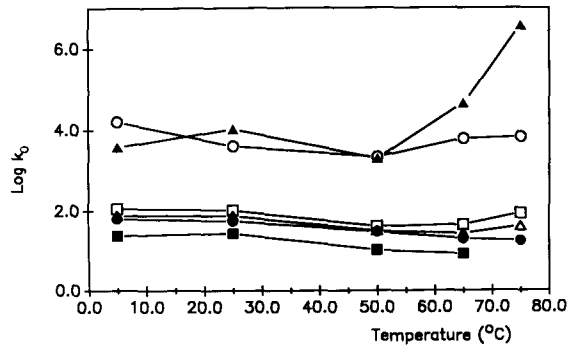

Fig. 2. Plot of log k_0 *versus* temperature (°C) for peptides **1–6** listed in Table I. See legend to Fig. 1 for experimental conditions. Peptides: (○), **1**; (●), **2**; (△), **3**; (▲), **4**; (□), **5**; (■), **6**.

apply to other well-manufactured *n*-alkylsilica stationary phases even in situations when small batch to batch variability may occur.

If a peptide existed in solution in an amphipathic helical conformation, then significant changes in the interactive properties would be anticipated as the operating temperature approaches and exceeds the melting temperature of an α-helix in the range 55–65°C[8]. As is evident in Fig. 1, the largest change in the S value over the range of temperatures was observed for $(Phe)_5$, which increased from 8 to 19.5 between 50 and 75°C, respectively. This clearly corresponds to the thermal denaturation of the peptide and was also associated with a significant increase in the log k_0 value (Fig. 2). These observations are a result of an increase in the number of residues that can simultaneously interact with the column when the peptide exists as the random coil structure. Of the remaining peptides, only the β-linked hGH peptide showed any significant increase in S value at the higher temperature. The S values for all other solutes remained essentially constant over the entire range of temperatures. This indicates that the α- and imide forms are more conformationally stable than the β-linked peptide. Detailed analysis of 2D-NMR NOESY spectra of the imide form[5] has revealed the existence of hydrogen bonding between the imide ring and the arginyl residue and the formation of the hydrophobic surface on one face of the helix. Preliminary evidence also suggests that the α-linked peptide also exists as an amphipathic helix, but the presence of the aspartyl residue results in an ionic interaction between the aspartyl carboxyl group and the arginyl residue. Clearly these interactions have resulted in a highly stabilised structure, while the additional carbon atom in the peptide backbone of the β-peptide appears to destabilise the amphipathic helix.

The ΔH^0_{assoc} and ΔS^0_{assoc} values for the peptides **1–4** were determined by linear regression analysis of plots of log $\bar{k}$ (at $\bar{\psi} = 0.3$) *versus* $1/T$, shown in Fig. 3, according to eqn. 5. The resulting values are listed in Table II. Two values of ΔH^0 and ΔS^0, corresponding to a high-temperature and a low-temperature value, were obtained for the β-linked peptide. Negative ΔH^0 values, as observed for the peptides used in this study, indicate that heat is released upon adsorption of the solute on the stationary phase. Positive values of ΔS^0 indicate that there is an increase in the disorder of the

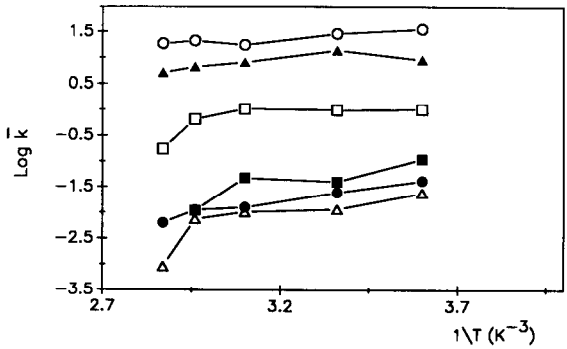

Fig. 3. Plot of log $\bar{k}$ versus $1/T$ (Van't Hoff plot) for peptides **1–6**, used to derive ΔH^0_{assoc} and ΔS^0_{assoc}, which are listed in Table III. Experimental log $\bar{k}$ values were derived at $\bar{\psi} = 0.3$. See legend to Fig. 1 for experimental conditions. Peptides: (○), **1**; (●), **2**; (△), **3**; (▲), **4**; (□), **5**; (■), **6**.

TABLE II

THERMODYNAMIC DATA FOR PEPTIDES SEPARATED ON THE C_{18} COLUMN

Peptide[a]	ΔH^0_{assoc} (*kJ mol*$^{-1}$)	ΔS^0_{assoc} (*J mol*$^{-1}$)
1 Imide	−3.3 ± 0.6	3.8 ± 0.6
2 α	−8.7 ± 0.3	−39.7 ± 1.2
3 β		
high T	−35.4 ± 8.9	−122.2 ± 30.6
low T	−6.1 ± 1.0	−32.8 ± 5.5
4 $(Phe)_5$	−3.3 ± 0.6	2.6 ± 1.1

[a] See Table I for amino acid sequence.

system during adsorption; two positive ΔS^0 values were observed for the imide and $(Phe)_5$. However, negative values were obtained for the α- and β-linked peptides. This indicates an increased ordering of the overall system. These results suggest that the open-chain peptides exist in solution in more flexible conformations, while the helical structure of the imide and $(Phe)_5$ are more rigid and constrained. This conclusion is also consistent with the COSY and NOESY NMR data[5]. The enthalpy and entropy values obtained in this study are also in the same range as other peptides including paracelsins[9] and insulin[10] which undergo conformational changes at the stationary phase.

The contribution of the hydrophobic ligands at the stationary-phase surface to conformational stability of the peptides was also investigated by comparing the chromatographic properties on the Poly F stationary phase. This packing material is a hydrophobic, polymeric fluorocarbon resin which does not contain the long alkyl-bond chains associated with more conventional reversed-phase materials. The S and $\log k_0$ values obtained for peptides **1–5**, separated on the Poly F column at 5, 25, 50, and 65°C, are listed in Table III. There was much more variability in both sets of values on the Poly F than observed with the octadecylsilica packing. In particular, there was a large change in the S value for the α-, and β-peptides between 5 and 25°C, followed by a large increase between 50 and 65°C for the open-chain peptides. This was associated with a steady decrease in the $\log k_0$ values of the α- and β-linked forms over

TABLE III

S AND LOG k_0 VALUES OBTAINED AT DIFFERENT TEMPERATURES WITH THE POLY F COLUMN

Peptide[a]	*5°C*		*25°C*		*50°C*		*65°C*	
	S	$\log k_0$	S	$\log k_0$	S	$\log k_0$	S	$\log k_0$
1 Imide	8.1	3.3	8.8	3.7	7.7	3.3	8.9	3.5
2 α	15.8	1.6	8.7	1.3	9.7	1.2	21.8	0.8
3 β	17.4	1.5	8.2	1.2	9.2	1.1	22.5	1.1
4 $(Phe)_5$	5.5	2.5	11.8	3.8	9.4	3.1	11.5	3.2
5 Phe	20.2	2.5	12.0	1.6	6.9	1.3	12.0	1.2

[a] See Table I for amino acid sequence.

the experimental temperature range while the value of S and log k_0 of the imide remained essentially constant. Clearly, the difference between the C_{18} ligands and the polymeric fluorocarbon surface is manifested as significantly altered interactive properties for these peptides. In particular, the high S values at low temperatures suggests that these peptides may have been trapped in the random-coil structure on the Poly F packing. As the temperature increases, the amphipathic helix can form, which then starts to unfold between 50 and 65°C. Furthermore, the S value for $(Phe)_5$ increased between 5 and 25°C and remained essentially constant at the higher temperatures. This contrasts with the results obtained for the octadecylsilica packing where increases in S value were observed between 50 and 65°C. This increase in S values, which is associated with helix denaturation at the lower temperature, indicates that the octadecylsilica packing material enhanced the stability of the $(Phe)_5$ helix. Similar ligand-induced behaviour is evident in stabilisation of the amphipathic helix of the hGH peptides presumably through interaction with the hydrophobic pocket containing Leu^1, Leu^4 and Phe^5. These results are also relevant to the known pattern of biological activity of these peptides. It has been suggested[11] that the planar surface on one side of the molecule in which the imide ring is located, is the active side of the molecule in its role as an insulin-potentiating agent. The interaction of the hydrophobic face on the alternate side of the molecule with a complementary surface at the site of biological recognition may serve to anchor the peptide specifically at the receptor surface. Thus, while the α- and β-forms, which are inactive in the glucose utilisation assay, may be able to bind to the biological recognition surface, the absence of the precise structural factors provided by the correct juxtapositioning of the arginyl-imide moieties prevents the open-chain peptides from exhibiting any biological activity.

The analysis of chromatographic retention data obtained at different operating temperatures provides significant insight into the influence of both solute structure and the nature of the stationary-phase ligand on the preferred orientation of peptide solutes at the hydrocarbonaceous surfaces used in RP-HPLC. By analogy, similar methods are also pertinent to the manner in which peptides interact with both synthetic and biological surfaces. The present results also demonstrate the utility of RP-HPLC techniques in providing information on peptide topography which is clearly important in the rational design and development of therapeutic analogues to naturally occurring peptide hormones.

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