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

XC[a]. INVESTIGATIONS INTO THE RELATIONSHIP BETWEEN STRUCTURE AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY RETENTION BEHAVIOUR OF PEPTIDES RELATED TO HUMAN GROWTH HORMONE

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SUMMARY

The gradient elution behaviour of eight synthetic peptides encompassing residues [6–13] of human growth hormone, *i.e.* Leu[1]-Ser-Arg-Leu-Phe-Asp-Asn-Ala[8], has been investigated, by using an octadecylsilica, a butylsilica, and a polymeric fluorocarbon as stationary phases. Quantitative expressions, derived from the linear-solvent-strength theory and the general plate-height theory, were used to assess the influence of gradient time on the relative retention and bandwidths of these peptides. It was demonstrated that the chromatographic properties of the cyclised imide form involving Asp[6] are consistent with the formation of a highly stabilised amphipathic helix, while the open-chain α- and β-rearranged forms eluted as less rigid structures. The putative hydrophobic contact region consists of two leucine residues and one phenylalanine residue. From an analysis of the retention and bandwidth data obtained at pH 9, a surface-induced molecular reorientation of the β-linked peptides was observed, in which the repulsion of the aspartyl carboxyl group from the hydrophobic stationary phase directs the C-terminal moiety away from the sorbent surface. Furthermore, the fluorocarbon sorbent exhibited characteristics favourable for use in preparative purification of these peptides. The present results demonstrate the sensitivity of reversed-phase high-performance liquid chromatography (RP-HPLC) to monitor small changes in the interactive behaviour of peptides with hydrocarbonaceous ligands and aquo-organic solvent combinations in reversed-phase systems. These observations further illustrate the general utility of HPLC for investigating the conformational behaviour of peptides at solid–liquid interfaces.

[a] For Part LXXXIX see ref. 20.

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INTRODUCTION

Human growth hormone (hGH) is a member of the somatotropic family of structurally conserved pituitary and placental proteins, important for the regulation of growth and lactogenesis in mammals. Typically, these proteins contain *ca.* 190 amino acid residues with molecular weights of *ca.* 22000. GHs exhibit several important physiological properties including the well-established growth-promoting activity, as well as significant effects on protein, lipid and carbohydrate metabolism. Numerous studies have been directed towards elucidating the relationship between the chemical structure and biological activity or immunoactivity of hGH[1,2]. In addition to the classical somatotropic and lactogenic activities of hGH, structure–function correlations have also been carried out by examining the biological activity of enzymatically and chemically modified derivatives, and by determining the relative affinities of hGH and its derivatives in radioimmuno assays and radioreceptor assays. Following the early report[3] that hGH, partially digested with chymotrypsin, retained significant somatotropic activity, considerable effort has been directed towards identifying a small fragment of hGH which retained potent biological activity[4,5]. To date this approach has been unsuccessful in segregating the somatotropic or lactogenic activities within the sequential structure of the protein. However, recent studies[6] on the hypoglycaemic and hyperglycaemic activities of hGH have identified an eight-residue segment at the amino terminus (residues [6–13]), which retains significant biological activity in terms of its insulin-potentiating properties. This sequence segment is located at the beginning of the first α-helical region (helix 1) of the protein, as predicted from our secondary-structure analysis[7] and confirmed by comparison with the recently reported X-ray structure of porcine GH[8]. Furthermore, it was found[9] that the cyclisation of the aspartyl residue at position 11 in the hGH sequence to give the imide derivative, is important for this biological activity. Two-dimensional NMR (COSY and NOESY) studies[9] of synthetic analogues to the peptide [6–13] have revealed that in solution the first five residues exist as an amphipathic helical structure which is then stabilised by the imide function.

The differences in the biological activity of the imide and the open chain α- and β-rearranged forms is related to the differences in molecular charge and conformation at the receptor surface[9]. Reversed-phase high-performance liquid chromatography (RP-HPLC) has been utilised in this laboratory as a physicochemical tool for the study of peptide behaviour at hydrophobic liquid–solid interfaces which mimic biological lipid bilayers. In particular, correlation of changes in retention and bandwidth behaviour over a range of chromatographic conditions with differences in primary and secondary structure has enabled us in earlier investigations to identify and characterise both the hydrophobic interaction sites and the existence of conformational equilibria with, for example, β-endorphin[10,11], luteinizing hormone-releasing hormone (LHRH)[12] and myosin kinase analogues[13]. The present paper provides a detailed analysis of the gradient elution behaviour of a set of eight peptides related to residues [6–13] of hGH. In particular, the retention and bandwidth behaviour under different pH values of the mobile phase and with different stationary phases has been analysed in terms of the relationship between molecular structure, hydrophobic contact area, and the predicted surface accessibility of the constituent amino acids. This approach also provides further insight into the important role that RP-HPLC data can play in

the design of peptide analogues of naturally occurring, biologically significant peptide hormones.

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 6-port HPLC 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 a Spectra-Physics SP4100 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.). Reversed-phase chromatography was carried out with a Bakerbond widepore octadecylsilica and butylsilica stationary phase (J. T. Baker, Phillipsburg, NJ, U.S.A.), both with a nominal particle diameter of 5 μm and average pore size of 30 nm, packed into 25 × 0.46 cm I.D. columns, 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 × 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.), trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, U.S.A.), and ammonium hydrogen carbonate (Bicarb; AnalaR) was obtained from BDH (Poole, U.K.). Water was distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The peptide analogues were synthesised by established F-moc procedures and purified by RP-HPLC, by using semi-preparative TSK C_{18} columns (250 × 10 mm) eluted with a gradient of 0–65% water in acetonitrile containing 0.1% TFA of 30 min duration and flow-rate of 2 ml/min. Purity , as assessed by amino acid compositional analysis, two-dimensional paper electrophoresis, fast atom bombardment mass spectrometry (FAB-MS) and multidimensional RP-HPLC, for the synthetic peptides was >95%.

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 (eluent A) and 0.1% TFA in 50% aqueous acetonitrile (eluent B) over gradient times varying between 15 and 90 min. The influence of basic conditions on peptide retention was studied by using a linear gradient from 25 m*M* Bicarb in water (eluent C) and 35 m*M* Bicarb in 50% aqueous acetonitrile (eluent D). Peptide solutions were prepared by dissolving the peptides in the appropriate eluent (A or C) at a concentration of 1 mg/ml, and sample sizes varied between 5 and 20 μg. 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[10,11]. Iterative regression analyses were performed by statistical

packages on a Monash University Computer Centre VAX11780 mini-computer system.

RESULTS AND DISCUSSION

Retention and bandwidth relationships

Expressions based on the linear-solvent-strength (LSS) gradient model have provided a useful approach for investigating the intimate relationship between the chromatographic behaviour of peptides and proteins and their primary, secondary, and tertiary structures. A detailed description of the application of the LSS theory to peptide separations in RP-HPLC has been outlined previously[10,14]. Briefly, the retention time, t_g, for a peptide, separated under ideal LSS conditions, can be used to determine the gradient steepness parameter, b, through the expression

$$b = t_0 \log \beta/[t_{g1} - (t_{g2}/\beta) + t_0 (t_{G1} - t_{G2})/t_{G2}] \quad (1)$$

where t_{g1} and t_{g2} are the solute gradient retention times at gradient times t_{G1} and t_{G2}, respectively, β is the ratio of the gradient times (t_{G2}/t_{G1}), and t_0 is the column dead-time. Evaluation of b values from retention data then allows the calculation of the median capacity factor, $\bar{k}$, and the corresponding organic mole fraction, $\bar{\psi}$, according to the relationships

$$\bar{k} = 1/1.15\, b \quad (2)$$

$$\bar{\psi} = [t_{g1} - t_0 - (t_0/b) \log 2]/(t_G/\Delta\psi) \quad (3)$$

Under regular reversed-phase conditions, $\bar{k}$ and $\bar{\psi}$ can be empirically related through the expression

$$\log \bar{k} = \log k_0 - S\bar{\psi} \quad (4)$$

The S and $\log k_0$ values can then be obtained by linear regression analysis of plots of $\log \bar{k}$ *versus* $\bar{\psi}$. The value of the parameter S in RP-HPLC is related to the magnitude of the hydrophobic contact area and the number of interaction sites, established between the solute and the stationary phase ligands during the adsorption process. Furthermore, the magnitude of the $\log k_0$ value is a measure of the free-energy changes associated with the binding of the solute to the stationary phase in the absence of the organic modifier. The determination of these parameters for a range of closely related peptides under a range of chromatographic conditions therefore provides the basis for quantitative characterisation of peptide orientation at the stationary phase surface.

Further characterisation of the interactive properties of peptides at hydrophobic surfaces can be carried out through the analysis of bandwidth dependencies. The relationship between the bandwidth, σ_v, and $\bar{k}$ for linear solvent systems can be expressed, according to the general plate-height theory, by

$$\sigma_{v,calc} = [\bar{k}/2) + 1]GV_m N^{-0.5} \quad (5)$$

where G is the band-compression factor, which arises from the increase in solvent strength across the solute zone as the gradient develops along the column, and N is the plate number. The derivation of eqn. 5 is based on the assumption that the shape of the peptide solute of a defined molecular weight can be characterised in terms of a constant hydrodynamic shape throughout the chromatographic separation. Under ideal conditions, the normalised ratio of the experimentally observed bandwidths to the calculated bandwidths ($\sigma_{v,exp}/\sigma_{v,calc}$) should approach unity over the normal operational range of $\bar{k}$ values. However, slow, time-dependent solvent- or stationary-phase-induced changes in the secondary or tertiary structure of the peptide solute will lead to significant alterations in surface topography or molecular dimensions of the peptide. This behaviour results in changes in the diffusional or interactive properties of the solute, such that experimentally observed peak widths will be significantly different from those predicted by eqn. 5.

As part of further investigations on the relationship between peptide structure and retention behaviour in RP-HPLC, gradient elution data were collected for a series of eight synthetic peptides, related to the N-terminal region (Leu^6–Ala^{13}) of hGH, with the view to provide further supportive information on the secondary and tertiary structure of these peptides, both in solution and at a hydrophobic surface.

Dependence of peptide retention and bandwidth on the stationary phase ligand

Based on extensive *in vitro* and *in vivo* biological evaluation of families of synthetic peptides related to hGH residues [6–13][9], a set of peptide analogues with known biological activities was selected for these investigations. The amino acid sequences of these peptides are listed in Table I, and are related to the linear peptide corresponding to residues [6–13] of hGH, *i.e.* Leu^1-Ser-Arg-Leu-Phe-Asp-Asn-Ala^8. The peptide analogues contain substitutions Asp^6→Glu^6, addition of a C-terminal Gly, replacement of the L-amino acid at position Leu^4 by the D-isomer or deletion of the amino-terminal Leu-Ser-Arg residues. The peptides are also characterised by the configuration of the side chain at position Asp^6, which exists as the naturally occurring

TABLE I

SEQUENCE, S, AND LOG k_0 VALUES FOR PEPTIDES USED IN THIS STUDY

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.

Peptide No.	*Sequence*	*Link*	C_{18}/0.1% TFA		C_4/0.1% TFA		Poly-F/0.1% TFA		C_{18}/Bicarb	
			S	*Log* k_0	*S*	*Log* k_0	*S*	*Log* k_0	*S*	*Log* k_0
1	LSRLFDNA	β	13.1	2.3	12.8	1.8	9.2	1.6	10.1	2.5
2	LSRLFDNA	imide	7.2	3.6	7.8	3.5	8.8	3.7	6.3	3.0
3	LSRLFENAG	β	12.0	2.1	13.1	1.9	8.1	1.5	8.1	2.1
4	LSRLFENAG	α	11.4	3.0	11.1	2.5	7.6	1.9	–	–
5	LSRL[a]FDNA	imide	8.2	4.0	7.3	3.2	7.1	3.1	7.4	3.7
6	LFDNAG	α	11.2	1.8	12.7	1.4	8.7	1.3	13.0	2.5
7	LSRLFDN-G	β	12.8	1.7	10.6	1.0	10.5	1.1	8.3	1.8
8	LSRLFENAG	β	12.8	1.9	13.0	1.5	8.2	1.2	9.5	1.8

[a] D-amino acid.

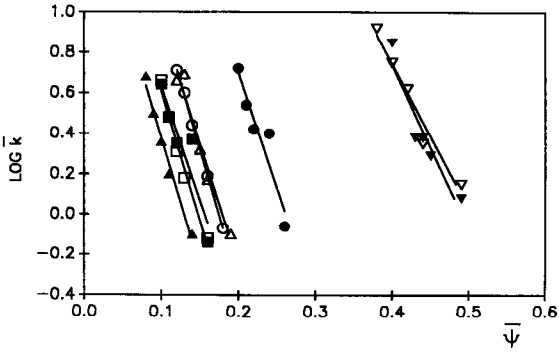

Fig. 1. Plots of log $\bar{k}$ *versus* $\bar{\psi}$ for hGH-related peptides **1–8**, separated on the C_{18} column at pH 2. The plots were derived from linear regression analysis of the data points (r^2 = 0.95–0.99), where t_G = 15, 30, 45, 60, and 90 min, and the flow-rate = 1 ml/min. See Table I for the derived S and log k_0 values and the Material and Methods section for other experimental details. Peptides: (○), **1**; (▽), **2**; (△), **3**; (●), **4**; (▼), **5**; (■), **6**; (▲), **7**; (□), **8**.

α-linkage, the cyclised imide form, or the β-linked peptide, which represents the alternative product obtained from imide cyclisation and subsequent hydrolysis. Peptides **1–8** were chromatographed, in the first instance, on a widepore octadecyl-silica (C_{18}), by using a linear gradient from 0.1% TFA in water to 0.1% TFA in 50% aqueous acetonitrile. Chromatographic data were accumulated for t_G = 15, 30, 45, 60, and 90 min at a fixed flow-rate of 1 ml/min.

Fig. 1 represents plots of log $\bar{k}$ *versus* $\bar{\psi}$ for the GH peptide analogues separated on the C_{18} column, and shows that essentially linear dependencies of log $\bar{k}$ *versus* $\bar{\psi}$ were observed, with correlation coefficients for linear regression between 0.95 and 0.99. The corresponding S and log k_0 values are also listed in Table I. Even with this set of closely related peptides, in which the amino acid compositions vary only slightly, it can be seen that selectivity changes occur over the range of experimental conditions used. The dependence of peptide retention on eluotropic strength in RP-HPLC can be categorised[15] on the basis of the relative S and log k_0 values. Thus, while large log k_0 values will be observed for hydrophobic peptides and lower values for polar peptides, both classes of solutes can exhibit a range of S values. The present results therefore represent examples that are consistent with our earlier conclusions[15] on structure–retention relationships for peptides in RP-HPLC as manifested by the log k' *versus* ψ dependencies. In particular, the imide-containing analogues exhibited higher log k_0 values than the α- and β-peptide structures. On superficial inspection, based solely on the linear amino acid sequence without consideration of the hierarchical structure of the peptides, it could be concluded that cyclisation of the β-carboxyl group of the aspartyl residue to form the imide, results in decreased molecular polarity and would be expected to result in increased retention. However, as is evident from Table I, the S values for the imides (*e.g.* peptides **2** and **5**) were significantly smaller than those for the corresponding α- and β-forms. This suggests that the hydrophobic contact area is much smaller for the imides than for the open-chain peptides. The chromatographic results for these peptides are fully consistent with the formation of an amphipathic helix, as depicted in Fig. 2 for the naturally occurring sequence of [6–13] in hGH. This

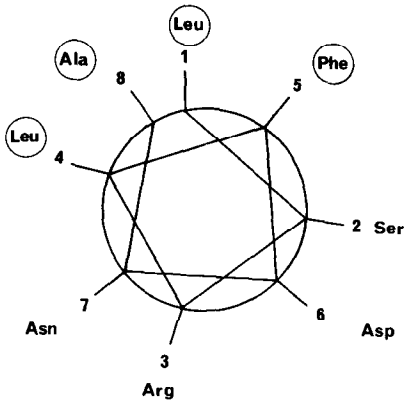

Fig. 2. Representation of the naturally occurring sequence of residues 6–13 of hGH on an Edmundson helical wheel.

helical structure results in the clustering of the hydrophobic residues Leu[1], Leu[4], Phe[5], and Ala[8] on one face of the helix of the open-chain peptide. This portion of the molecule would clearly represent the dominant interactive area in a reversed-phase system and would result in a high affinity for the hydrophobic ligands. The presence of a helical structure in solution has also been confirmed for the imide by two-dimensional NMR[9]. However, the formation of the imide bond between Asp[6] and Asn[7] inhibits extension of the helical structure through to the carboxy-terminal residues in the sequence and directs the Ala[8] residue away from the hydrophobic region of the peptide surface. The hydrophobic contact area of the imide peptides will therefore be diminished, as evidenced by lower S values. Therefore, the differences in the log k_0 values between the imides and the open chain peptides cannot be attributed to the relative polarity of the imide *versus* the aspartyl carboxyl group. Rather, two-dimensional NMR analysis[9] has revealed that the imide exerts an electron-polarising effect on the adjacent phenylalanine residue, thereby influencing the hydrophobicity of the interactive region of the peptide surface. Such constrained alignment of the hydrophobic residues on one face of the molecule clearly has important consequences as far as biological activity is concerned[9], *i.e.* the imide form of the peptide exhibits full activity in the glucose utilisation assay, whilst the α- and β-forms either lack activity or have very low activity. Generally, the β-peptides, which contain an additional methylene carbon in the peptide backbone, exhibited higher S values than the α-analogues but similar log k_0 values. Thus, it appears that the extra carbon atom has either been incorporated into the hydrophobic contact area or has caused a shift in the spatial disposition of the hydrophobic residues. The substitution of L-leucine for D-leucine (peptides **2** and **5**) did not result in any significant changes in the S and log k_0 values with this stationary phase. This suggests that the relative orientation of Leu[4] in the helical imide structure does not alter the interactive properties of the peptide. Furthermore, the substitution of Gly for Ala at position 8 (peptides **1** and **7**) resulted in a decreased log k_0, while the S value for these peptides was unaltered. This observation reflects the relative hydrophobicity of the two amino acids side chains and confirms the contribution of residue 8 to the retention process.

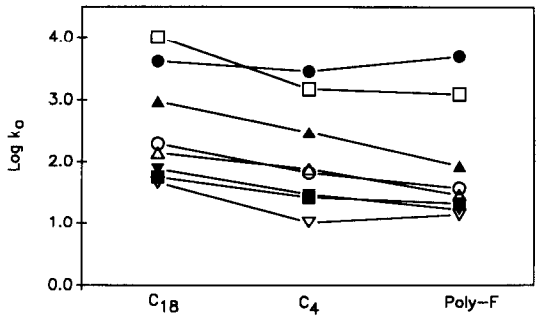

Fig. 3. Plot of log k_0 *versus* stationary phase ligand. See Table I for the log k_0 values. Peptides: (○), **1**; (●), **2**; (△), **3**; (▲), **4**; (□), **5**; (■), **6**; (▽), **7**; (▼), **8**.

There are several examples[16–18] where the presence of lipids have been found to enhance the formation and stability of amphipathic peptide helices in solution. Clearly, the nature of the hydrophobic C_{18} ligand may play a significant role in the equilibrium between the random coil and the helical structure of the GH-related analogues. Peptides **1**–**8** were therefore chromatographed on an *n*-butylsilica (C_4) stationary phase and a polymeric fluorocarbon (Poly F) packing material. The Poly F stationary phase is also a hydrophobic surface, yet it lacks the long carbon chains normally associated with reversed-phase packings. The log k_0 and S values obtained on both columns are listed in Table I and are also plotted against ligand type in Figs. 3 and 4. The log k_0 values for all peptides were lower on the C_4 than on the C_{18} column. However, while the S values for five of the peptides did not change significantly with the decrease in alkyl chain length, the S value on the C_4 phase increased for peptides **3** and **6** and decreased for peptide **7** relative to the results obtained on the octadecylsilica stationary phase. Hence, the different alkyl chain lengths change the way in which these three peptides interact with the surface. Previous studies[19] on the RP-HPLC of a group of peptides related to the paracelsins, separated on a series of sorbents with different alkyl chain lengths, revealed a discontinuity in the dependence of k' on the hydrophobic ligand carbon number when the chain length was C_4. It was postulated that the butyl chain existed as a more rigid unit than the longer chains and

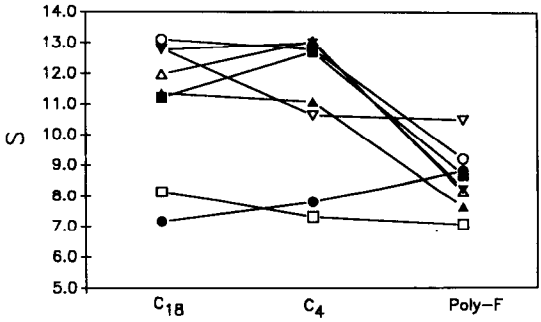

Fig. 4. Plot of log S *versus* stationary phase ligand. See Table I for the S values. Peptides: (○), **1**; (●), **2**; (△), **3**; (▲), **4**; (□), **5**; (■), **6**; (▽), **7**; (▼), **8**.

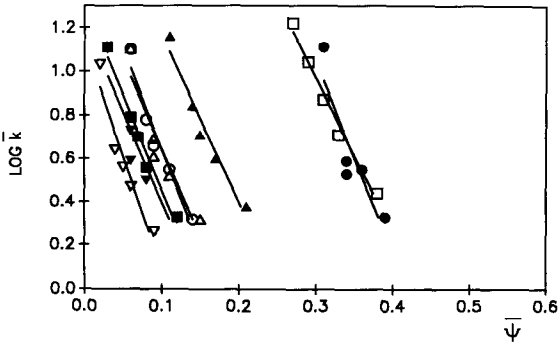

Fig. 5. Plots of log $\bar{k}$ *versus* $\bar{\psi}$ for peptides **1–8**, separated on the Poly F column at pH 2. See Fig. 1 for other experimental details. Peptides: (○), **1**; (●), **2**; (△), **3**; (▲), **4**; (□), **5**; (■), **6**; (▽), **7**; (▼), **8**.

induced some degree of preferred secondary structure in the peptide solutes. Clearly, a similar phenomenon could also be occurring with peptides **3**, **6** and **7**.

Plots of log $\bar{k}$ *versus* $\bar{\psi}$ for peptides **1–8**, separated on the Poly F column, are shown in Fig. 5. The S and log k_0 values for the imides (peptides **2** and **5**) when eluted from the octadecylsilica of Poly F sorbent essentially remained constant demonstrating that the interactive properties of these solutes with the Poly F sorbent are similar to that observed with the alkylsilica materials. Furthermore, these results indicate that the stability of the imide helix is unaffected by the presence or absence of alkyl chains bonded to the stationary phase surface. Conversely, both the S and log k_0 values for the α- and β-peptides decreased significantly with the Poly F column. These results indicate that a much smaller proportion of these peptides is involved in a weaker interaction with the fluorocarbon surface. The data also suggests that the interactive properties of the open-chain α-helix are strongly influenced by the physicochemical characteristics of the packing material, and further demonstrates the decreased conformational stability of these peptides relative to the imide-containing structures. These results are consequently consistent with the other observations and findings that the imide form of these peptides is fully constrained as a amphipathic helix whilst the α- and β-forms have significant flexibility in solution but can be induced to form a pseudo-helical conformation by an appropriate hydrophobic ligand.

The observed retention behaviour characterised by low S and log k_0 values for the Poly F column also represent properties suitable for the large-scale isolation of peptides and proteins. Under these conditions, lower amounts of organic solvent are required for elution and this, in the case of proteins, also minimises denaturation, reduces production costs, and increases the recovery of biological activity. While quantitative analysis by HPLC requires high peak capacity for all the components of interest, the preparative separation and recovery of pure compounds does not. In the present study, broader peak widths were generally observed on the Poly F phase than on the C_{18} material *e.g.* peak widths were approximately two-fold larger when comparable gradient conditions were employed. Under these circumstances, as long as the fraction collected between overlapping bands remains relatively small, peptides could be resolved to high purity. Thus, in addition to the increased pH stability of the stationary phase, the Poly F sorbent has significant potential for use in preparative HPLC.

Dependence of peptide retention and bandwidth on mobile phase pH

Two-dimensional NOESY NMR[9] of both the α- and imide forms of the peptide corresponding to residues [6–13] of hGH indicates that an ionic interaction occurs between Arg^3 and Asp^6 in the α-form and an ion–dipole interaction between the imide function and Arg^3 in the cyclised form. Inspection of a space-filling three-dimensional molecular model of the β-peptide reveals that for the ionic interaction to occur, a more strained configuration is required. As these interactions will be dependent on pH, the retention and bandwidth behaviour of peptides **1–8** at pH 2 (0.1% TFA) was compared with that at pH 9 (30 m*M* Bicarb) on the C_{18} stationary phase. The *S* and log k_0 values obtained are listed in Table I. The *S* and log k_0 values for the α-linked peptide **6** increased as the pH was increased. Thus, the ionisation of the aspartyl residue, which is located on the side of the helical structure directed away from the hydrophobic region, enhanced the affinity of the hydrophobic contact residues for the stationary phase ligands, presumably by bringing the Ala residue into closer proximity with the Leu and Phe residues. The most significant observation however, was a dramatic decrease in the *S* value for the β-linked peptides. Thus, as the peptide surface becomes more electrostatic due to potentially enhanced ion-bridging with the guanidinium group of the arginine residue or with the surrounding solvent, the COOH group effectively directs the C-terminal residues away from the stationary phase ligands, thereby reducing the interaction and decreasing the *S* value, *i.e.* the Ala^8 residue is no longer interacting with the hydrophobic stationary phase. This molecular reorientation at the surface appears to have relatively slow kinetics. Evaluation of the dependence of solute bandwidth on gradient steepness[11,12] for peptides **1–8** at pH 9 revealed that the bandwidth ratio for the β-peptides **1**, **3** and **7** were significantly higher with 30 m*M* Bicarb than with 0.1% TFA as the mobile phase, while the bandwidth ratio for the α- and imide peptides remained essentially constant.

The present study further demonstrates the utility of RP-HPLC for studying and characterising subtle changes in the interactive properties of biologically important peptides. Correlation of changes in the hydrophobic contact area and ligand affinity over a range of experimental conditions with differences in the amino acid sequence and composition has allowed the preferential orientational properties of these hGH-related peptides at hydrophobic surfaces to be studied. As documented elsewhere[9], the chromatographic properties of low *S* and high log k_0 values, which characterise the imide peptides, can be related to their biological potency as insulin-potentiating agents. The ability to correlate the influence of environmental and structural factors as monitored by chromatographic tools with the biological efficacy of peptides such as hGH residues [6–13] is thus expected to expand the utility of HPLC as a physicochemical technique in the design of peptide drugs and peptide derived vaccines.

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