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Comparison between the isocratic and gradient retention behaviour of polypeptides in reversed-phase liquid chromatographic environments ☆

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Abstract

The isocratic and gradient elution behaviour of β-endorphin and glucagon, two polypeptides known to exist in amphipathic α-helical conformations in lipophilic environments, have been examined under reversed-phase high-performance liquid chromatographic (RP-HPLC) conditions with low pH, aquo–acetonitrile mobile phases. The effects of changes in the volume fraction, ψ, of the organic solvent modifier and temperature, T, on the magnitudes of the S and $\log k_o$ values of these two polypeptides, obtained from the plots of logarithmic capacity factor ($\log k'$) vs. ψ using isocratic elution conditions have been determined. These data have then been compared to the corresponding $\bar{S}$ and $\log \bar{k}_o$ values, obtained from the plots of logarithmic median capacity factor ($\log \bar{k}$) versus the median volume fraction of the organic solvent modifier ($\bar{\psi}$) derived from the linear gradient elution data, using the same n-butyl silica sorbent and related aquo–acetonitrile mobile phase conditions. As apparent from these studies, substantial differences occur in the temperature-dependent trends and magnitudes of the corresponding S and $\bar{S}$ values, or the $\log k_o$ and $\log \bar{k}_o$ values, when these parameters are derived from experimental data acquired by these two different elution methods. Moreover, when gradient elution data for β-endorphin and glucagon are utilised, the extrapolated values of the intercept and slope of the plots of $\log \bar{k}$ vs. $1/T$ (corresponding to an apparent change in the median enthalpy of association, $\Delta \bar{H}^{o}_{assoc}$, or an apparent change in the median entropy of association, $\Delta \bar{S}^{o}_{assoc}$) substantially deviated from the values obtained for the thermodynamic parameters, ΔH^{o}_{assoc} and ΔS^{o}_{assoc}, derived from the $\log k'$ vs. $1/T$ plots using the corresponding isocratic data. These findings thus have important implications for biophysical and thermodynamic investigations when gradient elution data are employed to assess the molecular basis of the interaction of polypeptides with non-polar ligates. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Gradient elution; Mobile phase composition; Retention behaviour; Thermodynamic parameters; Endorphin; Glucagon; Peptides

1. Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) in the gradient elution mode represents a very powerful technique for the resolution of complex mixtures of polypeptides [1]. In recent years, various investigators have also applied gradient elution RP-HPLC procedures to examine the

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biophysical behaviour of polypeptides in hydrophobic environments. For example, RP-HPLC methods have been used in the characterisation of the conformational behaviour of these biosolutes [2–6], to assess the molecular nature of the hydrophobic binding site [7,8], or to extract hydrophobicity parameters related to the constituent amino acids [9–14]. Central to these considerations are the relationships that exist between the retention behaviour of polypeptides and the RP-HPLC experimental conditions, usually expressed in terms of the dependencies of the capacity factor, k' or in the case of gradient elution as a median capacity factor, $\bar{k}$ (see below for the equations linking k' and $\bar{k}$ to the experimentally determined isocratic and gradient retention times) on the volume fraction of the organic modifier, ψ, temperature, T, or other experimental variables, such as protonic ionisation or the solvent dielectric properties.

With isocratic elution systems, the relationship between $\log k'$ and ψ at a defined T over a wide range of ψ values, i.e. $\Delta\psi \geq 0.2$, usually takes the form of a U-shaped or hyperbolic dependency, the shape of which depends on the nature of the organic solvent modifier and the molecular characteristics of the polypeptide. For many polypeptides, over a narrower range of ψ values, i.e. $\psi \leq 0.2$, the relationship between $\log k'$ and ψ can be approximated as a linear dependency [15,16]. From practical considerations, primarily to accommodate the magnitude of the slope of these $\log k'$ vs. ψ dependencies, polypeptides are typically separated with RP-HPLC sorbents under low pH, linear gradient elution conditions using aquo–organic solvent mixtures. Gradient elution strategies provide efficient and highly reproducible methods well suited for the analytical and preparative separation of polypeptides. As a consequence, gradient elution RP-HPLC has become over the past 15 years or so, the dominant analytical and preparative separative procedure in laboratories interested in peptide synthesis and biology.

When RP-HPLC procedures are applied in other biophysical contexts, such as in the assessment of the magnitude of the change in Gibbs free energy, $\Delta\bar{G}^{o}_{assoc}$ associated with the interaction of a polypeptide with the solvated hydrocarbonaceous ligates, or in the derivation of extra-thermodynamic parameters, such as amino acid hydrophobicity parameters, structure–retention τ values or partition coefficients [15,17], additional considerations arise when gradient elution modes are employed. These considerations partly relate to the nature of the hierarchal secondary and higher order structures of polypeptides as well as the proclivity of these molecules to undergo multi-site interactions with immobilised hydrocarbonaceous ligands. Fundamental thermodynamic issues pertinent to the binding and desorption processes must be reconciled if gradient elution data are to be extrapolated and qualitatively used to assess the apparent free energy changes that characterise polypeptide–ligate interactions in response to changes in experimental conditions, such as changes in temperature or the surface density of immobilised n-alkyl chains with n-alkylsilica. Similar physicochemical considerations also apply when gradient elution data are used to evaluate the intrinsic hydrophobicity of a polypeptide or to assess the molecular features of the binding site and the role of conformational effects for a polypeptide in contact with non-polar ligates. For the derived thermodynamic or extra-thermodynamic parameters to thus have physical meaning when gradient measurements are employed with polypeptides, the requirement exists for the polypeptide–ligand–solvent complex as a ternary interactive system to have reached a series of instantaneous states, each approximating the near equilibrium condition. Equivalence in the trends of extra-thermodynamic terms, such as the gradient-derived $\bar{S}$ and $\log \bar{k}_o$ values with the corresponding S and $\log k_o$ values derived from isocratic measurements, is essential if gradient retention data are to be reliably correlated with linear free energy relationships or with molecular descriptor terms (e.g. sterimolar parameters, accessible surface area terms, hydrophobicity coefficients, etc.) of polypeptides in contact with non-polar ligates in a manner analogous to that used with the corresponding isocratic data.

With low-molecular mass neutral or polar organic compounds and small peptides, the available body of scientific evidence dating back to the mid/late-1980s favours [18–23] a reasonable equivalence in the values of some extra-thermodynamic parameters, such as hydrophobicity coefficients, when derived by either gradient or isocratic methods, both in terms of the magnitudes of their values and the trends that they follow on variation in the RP-HPLC conditions.

With polypeptides of size larger than 15–20 amino acid residues, the evidence supporting this equivalence is, however, far less complete or compelling. Surprisingly, few studies have examined in any depth the retention behaviour of the same polypeptides under notionally equivalent isocratic and gradient elution conditions in an otherwise identical RP-HPLC system. Accordingly, in this study the isocratic and gradient elution behaviour of the polypeptides, β-endorphin and glucagon, have been examined with the same *n*-alkylsilica and aquo–organic solvent systems. These polypeptides were selected because of their propensity to adopt α-helical conformations in lipophilic environments. In addition, they can be eluted over a similar range of organic solvent concentrations with RP-HPLC systems, despite their different amino acid sequences. As such, these polypeptides provide sensitive probes for the interaction with the same *n*-alkylsilica sorbent as the temperature of the system was adjusted with notionally equivalent mobile phase conditions under isocratic or gradient elution modes.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (HPLC grade) was obtained from either Mallinckrodt (Paris, KY, USA) or EM Industries (Gibbstown, NJ, USA) whilst trifluoroacetic acid (TFA, peptide synthesis grade) was purchased from Auspep (Parkville, Australia) or Pierce (Rockford, IL, USA). Water was distilled and deionised with a Milli-Q system (Millipore, Bedford, MA, USA). *N*-Acetyl-L-α-phenylalanine ethyl ester, *N*-acetyl-L-α-tryptophanamide and glucagon were obtained from Sigma (St. Louis, MO, USA), and were of >95% purity. The β-endorphin (purity also >95%) was obtained from Auspep and Organon (Oss, The Netherlands). Other reagents were obtained from Merck Australia (Kilsyth, Australia).

2.2. Apparatus

All chromatographic measurements were made on either a chromatographic system consisting of a Beckman System Gold autosampler 507, programmable solvent module 126, and programmable detector module 116, or a Perkin-Elmer (Norwalk, CT, USA) series 4 HPLC system. Temperature was controlled by either surrounding the column in a thermostatted water jacketed cylinder, coupled to a BioRad module 486 refrigerated recirculating bath (BioRad, Richmond, CA, USA) or to an ICI TCl900 HPLC oven (ICI Instruments, Dingley, Australia). The chromatographic measurements were performed with Bakerbond wide-pore *n*-butylsilica (J. T. Baker, Phillipsburg, NJ, USA) packed into columns with dimensions of 250×4.6 mm I.D. The RP-HPLC sorbent had a nominal particle size of 5 μm and a 30-nm average pore size. Columns from the same batch were used in order to minimise variations in the manufacture of the sorbents.

Circular dichroism measurements were carried out using an AVIV model 62DS CD spectrometer (Aviv Associates, Lakewood, NJ, USA). Wavelengths were scanned between 190 and 250 nm under constant flushing with high-grade nitrogen. The cell employed in all CD measurements had a path length of 1.0 mm. Data recording was computer-controlled, with the data points recorded at wavelength steps of 0.2 nm and a bandwidth of 0.8 nm by multiple scanning procedures ($n>5$). Multiple linear regression analysis was performed using the software PLOT with comparison to reference spectra for pure α-helix, β-sheet, β-turn and random coil structures. Peptide concentrations (as determined by amino acid analysis) were 500 μg/ml in (a) phosphate buffer, pH 2.20 (15 m*M* orthophosphoric acid, obtained from E. Merck, Darmstadt, Germany, titrated with 5 *M* sodium hydroxide); (b) trifluoroethanol–phosphate buffer, pH 2.20 (90:10); and (c) acetonitrile–phosphate buffer, pH 2.20 (40:60, v/v). The wavelength dependency of the mean molar residue ellipticity, $[\Theta]$ (degree cm^2 $dmol^{-1}$) was obtained from

$$[\Theta]=\frac{M_r\times\Theta}{Cl} \quad (1)$$

where M_r is the mean molecular mass, C is the concentration (g/ml), l is the path length (cm) and Θ is the ellipticity (degrees). In the case of the acetonitrile–phosphate buffer, pH 2.20 (40:60) solvent, absorption effects due to the solvent precluded accurate measurements of Θ at wavelengths below 195 nm. Both β-endorphin and glucagon at con-

centrations of ≤1 mg/ml in aqueous acetonitrile solutions exist as monomeric species.

2.3. Chromatographic procedures

Bulk solvents were filtered and degassed by sparging with nitrogen. Isocratic elution with mobile phases of different organic modifier volume fractions was performed using 0.1% TFA in acetonitrile–water with the acetonitrile composition varying by steps of 1% (v/v) from 25 to 30% (v/v) for β-endorphin and from 25 to 32% (v/v) for glucagon. The flow-rate was held at 1.0 ml/min throughout these investigations with the peak profiles monitored at 215 nm. Column temperatures were controlled at 5, 15, 25, 35, 45, 55, 65, 75 and 85°C and were accurate to ±0.5°C. Peptide solutions were prepared by dissolving the solute at a concentration of 500 μg/ml in 0.1% TFA. Injection size varied between 1 and 5 μg. All data points were derived from at least duplicated measurements with retention times between replicates typically varying by less than ±1%. The column dead volume was measured based on the retention time of the non-interactive solute, sodium nitrate. For the gradient measurements, solutions of β-endorphin and glucagon were prepared by dissolving the solute at a concentration of 100 μg/ml in 0.1% TFA (buffer A), whilst the injection size varied between 0.5 and 2 μg of polypeptide. All data points were derived from at least duplicate measurements with retention times between replicates varying less than ±0.5%. Linear gradient elution was performed using 0.1% TFA in water (buffer A) and 0.09% TFA in 65% aqueous acetonitrile (buffer B) over the gradient times of 15, 30, 45, 60, 75, 90, 120, 150 and 180 min at a flow-rate of 1 ml/min. Other procedures for the acquisition of the gradient elution chromatographic data were based on methods described previously [24,25].

Since prolonged use of *n*-alkylsilica columns at high temperatures can under some elution conditions have detrimental effects on the resolution, column capacity and peak shape of polypeptides, *N*-acetyl-L-α-phenylalanine ethyl ester and *N*-acetyl-L-α-tryptophanamide were run sequentially as control solutes with the test polypeptides, β-endorphin and glucagon. The retention and peak-shape characteristics of these low-molecular mass solutes were continuously monitored and when significant changes in these parameters were observed, the columns were replaced. Columns from the same batch were used in order to minimise the influence of variations in the manufacture of the sorbents.

2.4. Analysis of experimental data

Chromatographic retention time data obtained using isocratic procedures with the polypeptides was converted to the corresponding k' values utilising the conventional equation $k'=(t_e-t_o)/t_o$, where t_e and t_o are the elution time and the column void time, respectively. Similarly, the chromatographic parameters, S and $\log k_o$, obtained from the plots of $\log k'$ vs. ψ when isocratic elution data were utilised, or the $\bar{S}$ and $\log \bar{k}_o$ terms, derived from the plots of $\log \bar{k}$ vs. $\bar{\psi}$ when gradient elution data were employed, were calculated using the programs written for IBM-compatible personal computers [4,16,17], based on the considerations inherent to Eqs. (7) and (11). Statistical analysis of isocratic and gradient data used ANOVA regression analysis procedures, assuming first, second or higher order correlations between dependent parameters whilst confidence intervals of 95% were employed.

3. Results and discussion

3.1. Theoretical considerations

When the interaction of a polypeptide with the immobilised *n*-alkyl chains in a RP-HPLC system satisfies the criterion of near equilibrium in the elution process, then the capacity factor, k', can be related to the association constant, K_{assoc}, and to the change in Gibbs free energy for the polypeptide–ligate association, ΔG^o_{assoc}, through the relationships:

$$\log k' = \log K_{assoc} + \log \Phi \tag{2}$$

$$\log K_{assoc} = -\Delta G^o_{assoc}/RT \tag{3}$$

and thus

$$\log k' = -\Delta G^o_{assoc}/RT + \log \Phi \tag{4}$$

where R, T and Φ are the universal gas constant

($=8.21\times10^{2}$ l atm K^{-1} mol^{-1}; 1 atm=101325 Pa), the absolute temperature (K) and the phase ratio, respectively.

According to the Gibbs–Helmholtz relationship,

$$\Delta G^{\circ}_{\text{assoc}} = \Delta H^{\circ}_{\text{assoc}} - T\,\Delta S^{\circ}_{\text{assoc}} \quad (5)$$

where $\Delta H^{\circ}_{\text{assoc}}$ and $\Delta S^{\circ}_{\text{assoc}}$, respectively, are the enthalpy and entropy changes associated with the interaction of the polypeptide with the non-polar ligates during the chromatographic migration process. Hence, the dependency of k' on temperature can be represented in the form of the well-known classical Van 't Hoff equation, namely

$$\log k' = -\frac{\Delta H^{\circ}_{\text{assoc}}}{RT} + \frac{\Delta S^{\circ}_{\text{assoc}}}{R} + \log \Phi \quad (6)$$

Moreover, the dependency of the capacity factor, k', of a polypeptide on the volume fraction, ψ, of the organic solvent modifier in RP-HPLC under isocratic conditions can be approximated [1,16,17] over a limited range of ψ values by the empirical relationship:

$$\log k' = \log k_{o} - S\psi \quad (7)$$

where $\log k_{o}$ is the intercept value when $\psi\rightarrow0$, and S is the gradient of the plot of $\log k'$ vs. ψ. In a variety of investigations with polypeptides and proteins, reasonable correlations for a linear dependency between $\log k'$ and ψ over limited ranges of ψ values (i.e. when $\Delta\psi\leq0.15$) have been observed, although it can be noted that over wider ranges of ψ values (i.e. when $0.15\leq\Delta\psi\leq0.6$) curvilinear plots have typically been found for many peptides as well as higher molecular mass proteins. These observations are consistent with the interplay of second order interactive processes that are solute specific for a particular RP-HPLC sorbent or mobile phase condition [1,13,15,16,26,27]. Because the slopes (or gradients), S, of the $\log k'$ vs. ψ plots for polypeptides and proteins are typically large in RP-HPLC systems, this dependency results in a relatively narrow elution window over which the solute will migrate and satisfy the near-equilibrium assumptions of isocratic linear elution chromatography. Consequently, gradient elution conditions are frequently required with mixtures of polypeptides because of this profound dependency of $\log k'$ on ψ in order to permit generation of solute retention volumes corresponding to ≤20 column volumes. With gradient elution methods, the adsorption of the polypeptide is initially achieved with a water-rich eluent, typically involving aqueous 0.1% TFA, with elution affected by a binary or ternary organic solvent rich mobile phase, typically containing up to 60% (v/v) or a higher percentage of an organic solvent in a water–0.1% TFA mixture.

Under conditions of linear solvent strength changes in gradient elution, the migration of the solute can be described according to the linear solvent strength (LSS) model [16–18,28,29] in terms of the gradient steepness parameter, b, and the median capacity factor, $\bar{k}$, such that

$$b = \left[\frac{t_{o}\log\beta}{t_{g1} - \dfrac{t_{g2}}{\beta} + t_{h}\dfrac{(t_{G1}-t_{G2})}{t_{G2}}}\right] \quad (8)$$

and

$$\bar{k} = 1/1.15b \quad (9)$$

where t_{o} is the column void time, t_{h} is the system hold-up time for the buffer B to reach the head of the column after commencement of the gradient, t_{g1} and t_{g2} are the gradient elution times of the polypeptide under two different gradient times of duration t_{G1} and t_{G2}, respectively, and β is the ratio of the gradient duration times (i.e. $\beta=t_{G2}/t_{G1}$).

Similarly, the median volume fraction of the organic solvent modifier, $\bar{\psi}$, corresponding to a notional instantaneous value of the volume fraction of the organic solvent modifier at which the solute commences to be desorbed from the hydrocarbonaceous sorbent can be described according to the LSS model as

$$\bar{\psi} = \left[t_{g1} - th - \left(\frac{t_{o}}{b}\right)\log 2\right]\frac{\Delta\psi}{t_{G1}} \quad (10)$$

where the overall rate of change of $\Delta\psi$ for a gradient time of t_{G1} is given by $\Delta\psi/t_{G1}$.

Under experimental conditions of linear gradient slopes and linear solvent strength changes, the dependency between $\log\bar{k}$ and $\bar{\psi}$ for polypeptides can be represented by analogy with the isocratic situation in terms of the empirical relationship:

$$\log\bar{k} = \log\bar{k}_{o} - \bar{S}\,\bar{\psi} \quad (11)$$

where $\bar{S}$ is the slope (or gradient) and $\log \bar{k}_o$ is the extrapolated y-intercept at $\bar{\psi} \rightarrow 0$ of the $\log \bar{k}$ vs. $\bar{\psi}$ plot. Analysis of the experimental retention data for polypeptides in terms of this empirical relationship, as well as the corresponding dependency for the isocratic elution mode, provides a theoretical framework for ascertaining whether the same or similar phenomena are being monitored during gradient elution as occur under the near equilibrium conditions of isocratic elution. Thus, if the magnitudes and trends of the S and $\bar{S}$ values (or the $\log k_o$ and $\log \bar{k}_o$ values) as a function of temperature are identical or very similar within experimental error, conclusions can be reached on the physical significance of the gradient-derived parameters in terms of their interchangeability with the corresponding results obtained from isocratic data. If, on the other hand, differences arise between the S and $\bar{S}$ values (or the $\log k_o$ and $\log \bar{k}_o$ values) as a function of temperature, then elaboration of the molecular processes that occur during the interaction of a polypeptide with a reversed-phase sorbent with data derived from gradient measurements will result in physically less reliable outcomes compared to the information obtained from the corresponding isocratic data. When this latter circumstance prevails, significant quantitative and qualitative differences will emerge between the relationships of the S or $\bar{S}$ values and their molecular parameters such as the hydrophobic contact surface area, ΔA_{hyd}, established between the solute and the hydrocarbonaceous ligand at the surface of the RP-HPLC sorbent. Variations between the dependencies of $\log k'$, S or $\log k_o$ on temperature and the corresponding dependencies $\log \bar{k}$, $\bar{S}$ or $\log \bar{k}_o$ on temperature will similarly be diagnostic of conformational processes which may occur for a polypeptide during the chromatographic migration [1,5,7,16,23,30,31]. Such considerations are also be relevant if the evaluation of thermodynamic parameters, such as the median changes in enthalpy and entropy, $\Delta \bar{H}^o_{assoc}$ and $\Delta \bar{S}^o_{assoc}$ are contemplated from extrapolated gradient data, since the nature of these dependencies under temperature-dependent conditions will be different for isocratic elution procedures compared to the instantaneously changing solvent conditions that are generated during gradient elution.

With these considerations in mind, the retention behaviour of β-endorphin and glucagon have been investigated using notionally equivalent isocratic and gradient elution chromatographic conditions with particular reference to the magnitudes and trends in the S and $\bar{S}$ values, and the k_o and $\bar{k}_o$ values, as a function of temperature. The results below indicate that significant differences arise for these polypeptides in terms of the magnitude and trends of these extra-thermodynamic values when linear gradient and isocratic elution data are compared.

3.2. Dependency of log k′ on ψ and $\log \bar{k}$ *on* $\bar{\psi}$ *for β-endorphin and glucagon as a function of temperature, T*

The biological activity of the 31 amino acid residue neuropeptide, β-endorphin (amino acid sequence listed in the legend to Fig. 1a), is believed to involve the interaction of the N-terminal pentapeptide unit Tyr–Gly–Gly–Phe–Met with κ- or μ-opioid receptors with the C-terminal 19-mer segment from Pro^{13} assuming an amphipathic α-helix in the presence of the hydrophobic environment of the receptor [32–34]. A variety of investigations with synthetic variants of β-endorphin have documented that the extent of stabilisation achieved by this amphipathic α-helical structure determines not only the receptor specificity, but also the magnitude of regulation of the pain response associated with the function of these neuroactive polypeptides. Glucagon (amino acid sequence listed in Legend to Fig. 1b) is a 29 amino acid residue polypeptide which is also thought on the basis of high field ^{1}H-NMR spectroscopic studies [35] to assume an α-helical structure in lipophilic environments, with α-helices extending from Tyr^{10}–Leu^{14} and Arg^{17}–Thr^{29}, permitting this pancreatic polypeptide to elicit gluconeogenic responses with target cells by generating 'bioactive' conformations [36–38] with its membrane-associated receptors.

As is apparent from the circular dichroism (CD) spectra shown as Fig. 1a,b, β-endorphin and glucagon exhibit β-sheet but no α-helical secondary structure in an aqueous milieu such as 15 m*M* phosphate buffer, pH 2.20, (ca. 56 and 65% β-sheet content, respectively, based on multiple linear regression of the CD data). In the presence of 90% (v/v) trifluoroethanol, a α-helix-inducing solvent, con-

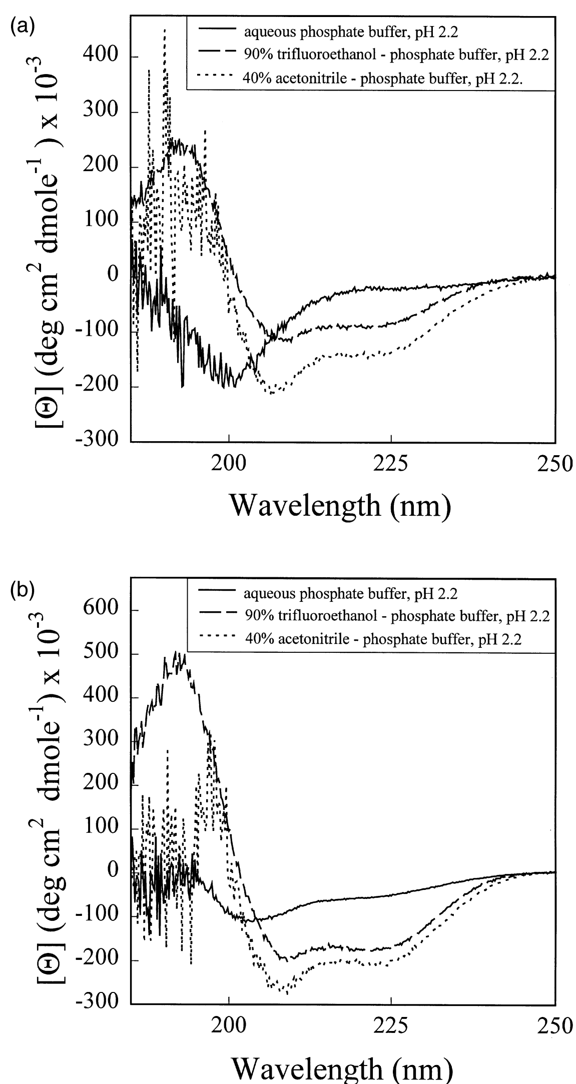

Fig. 1. Circular dichroism spectra for β-endorphin (a) and glucagon (b) measured in phosphate buffer (15 m*M* orthophosphoric acid, titrated with 5 *M* sodium hydroxide to pH 2.20); (b) trifluoroethanol–phosphate buffer, pH 2.20 (90:10); and (c) acetonitrile–phosphate buffer, pH 2.20 (40:60, v/v). Peptide concentrations were 500 μg/ml. Other conditions of these experiments are given in Section 2. The one-letter code for the amino acid sequence of β-endorphin is H_2N-YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE-OH and for glucagon is H2N-HSQGTFTSDYSKYLDSRRAQDFVQWLMNT-OH, respectively.

formational changes were induced resulting in these polypeptides assuming α-helical contents of ca. 29 and 65%, respectively. In the presence of 40% (v/v) acetonitrile, corresponding to an organic solvent percentage value slightly above that at which β-endorphin or glucagon elute from the *n*-butylsilica sorbent, significant α-helical contents (ca. 43 and 21%, respectively) were evident for both polypeptides. Thus, these polypeptides have the propensity to adopt α-helical structures under the bulk aquo–organic solvent conditions of reduced or low water content. By analogy also with structure–retention data [16,39] obtained for different structural analogues in lipophilic environments, the conclusion can be reached that β-endorphin and glucagon adopt and maintain more extensive α-helical structures on adsorption to the *n*-alkyl ligates of a RP-HPLC sorbent. These conformational structures progressively unfold as the temperature of the system is increased. Analogous observations have been obtained in RP-HPLC investigations and molecular mechanics–molecular dynamics simulation studies with the polypeptide bombesin [4,40].

In Fig. 2a,b are shown the plots of $\log k'$ vs. ψ and $\log \bar{k}$ vs. $\bar{\psi}$ for β-endorphin using the *n*-butylsilica sorbent and acetonitrile–water mobiles phases, pH 2.2 as the temperature was increased from 5 to 85°C. The derived values of S and $\log k_o$, or $\bar{S}$ and $\log \bar{k}_o$, are listed in Table 1, together with the correlation coefficient values, assuming that these dependencies satisfy the linear relationships given by Eqs. (7) and (11). Similarly, in Fig. 3a,b are shown the plots of $\log k'$ vs. ψ and $\log \bar{k}$ vs. $\bar{\psi}$ for glucagon obtained under similar conditions. The derived S, $\bar{S}$, $\log k_o$ and $\log \bar{k}_o$ values are listed in Table 1 together with the correlation coefficient values, assuming that these dependencies satisfy the linear relationships given by Eqs. (7) and (11). In Figs. 2a and 3a are also shown the regression lines and the 95% confidence limits for the isocratic data analysed in terms of a first order relationship between $\log k'$ and ψ. For reasons of clarity, only the regression lines have been shown in the corresponding plots of the gradient data (Figs. 2b and 3b) with the 95% confidence limits omitted.

3.3. Dependency of S or $\bar{S}$ on temperature and $\log k_o$ or $\log \bar{k}_o$ on temperature for β-endorphin and glucagon

As evident from Figs. 2a,b and 3a,b and the data listed in Table 1, significant differences occur be-

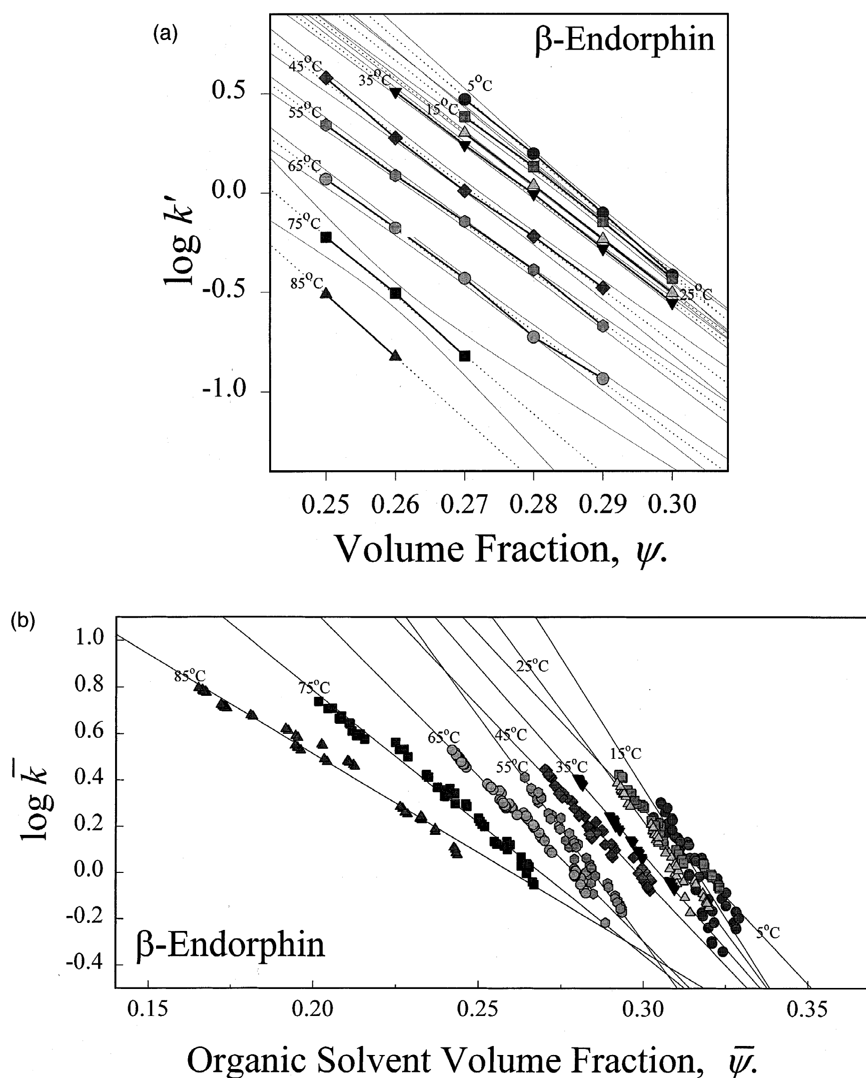

Fig. 2. (a) Plots of $\log k'$ vs. ψ for β-endorphin measured with a n-butylsilica reversed-phase sorbent at temperatures from 5 to 85°C under isocratic elution conditions at a flow-rate of 1 ml/min, encompassing the range of acetonitrile concentrations from $0.25<\psi<0.30$. Also shown in this figure is the linear regression line (dotted points) and the boundary lines for confidence intervals of 95% assuming that the dependency between $\log k'$ and ψ can be described in terms of Eq. (7). (b) Plots of $\log \bar{k}$ vs. $\bar{\psi}$ for β-endorphin measured with the same n-butylsilica reversed-phase sorbent at different temperatures from 5 to 85°C, encompassing the gradient time duration of 15, 30, 45, 60, 75, 90, 120, 150 and 180 min at a flow-rate of 1 ml/min, using linear gradient elution conditions generated from 0.1% TFA in water (buffer A) and 0.09% TFA in 65% aqueous acetonitrile (buffer B), corresponding to the notional range of acetonitrile concentrations of $0.16<\bar{\psi}<0.33$ for the elution of this polypeptide. Shown in this figure is the linear regression line, but the boundary lines for confidence intervals of 95%, assuming that the dependency between $\log \bar{k}$ and $\bar{\psi}$ can be described in terms of Eq. (11), have been omitted for reasons of clarity

tween the derived S and $\bar{S}$ values, or the $\log k_o$ and $\log \bar{k}_o$ values, over the temperature range studied. These results indicate that the magnitudes of these extra-thermodynamic parameters for β-endorphin and glucagon are dependent on whether they are derived from isocratic or gradient data. These differences reflect the influence of solvational effects on the conformational status of these polypeptides in the bound and free states as well as analogous effects on the immobilised chains of the non-polar ligate. For

Table 1
Comparative values of the S and $\log k_o$ values, and the corresponding $\bar{S}$ and $\log \bar{k}_o$ values, of β-endorphin and glucagon as a function of temperature, T, determined using experimental data derived from the isocratic and gradient elution measurements according to Eqs. (7) and (11). The correlation coefficients, r^2, were derived assuming that a linear dependency existed between $\log k'$ and ψ for the isocratic measurements and $\log \bar{k}$ and $\bar{\psi}$ for the linear gradient elution measurements. The one letter code for the amino acid sequence of β-endorphin is H_2N-YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE-OH and for glucagon is H_2N-HSQGTFTSDYSKYLDSRRAQDFVQWLMNT-OH, respectively

T (°C)	β-Endorphin						Glucagon					
	Isocratic			Gradient			Isocratic			Gradient		
	S	Log k_o	r^2	$\bar{S}$	Log $\bar{k}_o$	r^2	S	Log k_o	r^2	$\bar{S}$	Log $\bar{k}_o$	r^2
5	29.6	8.5	0.9989	22.4	7.07	0.7959	25.0	7.85	0.9998	18.0	6.24	0.8883
15	27.3	7.76	0.9952	15.04	4.78	0.9642	23.3	7.23	0.9999	13.8	4.76	0.9734
25	26.0	7.59	0.9999	19.09	5.94	0.9687	22.3	6.80	0.9998	17.2	5.74	0.9887
37	26.5	7.4	0.9996	16.2	4.92	0.9969	21.5	6.42	0.9997	15.3	4.94	0/9952
45	26.2	7.10	0.9977	14.89	4.48	0.9752	21.8	6.25	0.9973	14.1	4.42	0.9880
55	25.1	6.62	0.9989	19.37	5.51	0.9652	21.8	5.99	0.9989	17.3	5.12	0.9827
65	25.6	6.48	0.9981	14.26	3.98	0.9916	23.1	6.06	0.9974	13.7	3.93	0.9925
75	29.9	7.25	0.9990	11.4	3.06	0.9795	24.3	5.94	0.9983	11.6	3.17	0.9897
85	31.4	7.33	1.000d	8.54	2.22	0.9788	26.2	6.07	1.000	7.8	2.07	0.9792

example, when isocratic conditions are employed within the range $0.25<\psi<0.32$, adsorption of β-endorphin and glucagon in conformational states of significant α-helical content will be favoured, as indicated by the CD studies. With gradient elution, on the other hand, a continuum of solvation and desolvation changes will occur for both the polypeptide and the immobilised n-alkyl ligate as the gradient is developed. In the gradient mode, adsorption also occurs from an aqueous buffer A with β-endorphin and glucagon having the potential to exist in conformational states of lower α-helical content, but higher β-sheet content under these conditions [33,34] (cf. also Fig. 1). In turn, these processes are reflected as different dependencies of the respective capacity factors, k' and $\log \bar{k}$, on the solvent content and temperature. The magnitude of these differences in the slope and intercept values derived from the plots of $\log k'$ vs. ψ, and $\log \bar{k}$ vs. $\bar{\psi}$, for β-endorphin and glucagon as a function of temperature, and their trend lines, become particularly evident when the derived S and $\bar{S}$ values or the $\log k_o$ and $\log \bar{k}_o$ values are plotted as a function of temperature as shown in Figs. 4 and 5. Thus, when the S vs. T and the $\log k_o$ vs. T plots derived from the isocratic data for β-endorphin were analysed, curvilinear dependencies were observed as the temperature was increased from 5 to 85°C, with these dependencies essentially following second order relationships. The isocratic data for glucagon followed similar trends in terms of the relationship between S and T or $\log k_o$ and T. Although the magnitudes of the S (or $\log k_o$) values for β-endorphin and glucagon fell within a similar region, under all experimental conditions of T and ψ, the plots of S vs. T and $\log k_o$ vs. T for β-endorphin and glucagon were discrete, and did not show cross-over points or common values (Fig. 4). The trends evident in these plots in terms of decreasing S values over the temperature range of ca. 5–50°C followed by increasing S values at higher T was also reflected in the ability to resolve these polypeptides with the n-butylsilica sorbent under all isocratic conditions used in this investigation.

In contrast, the plots of $\bar{S}$ vs. T and $\log \bar{k}_o$ vs. T (Fig. 5) derived from the gradient data for β-endorphin and glucagon were curvilinear, requiring at least a third order function to enable adequate curve fitting. As noted above, the magnitude of the $\bar{S}$ and $\log \bar{k}_o$ values at all temperatures were smaller than those found for the corresponding S and $\log k_o$ values. The shapes of the plots of S vs. T and $\bar{S}$ vs. T for β-endorphin and glucagon followed different slope aspects, with similarly divergent slopes apparent for the corresponding comparisons of $\log k_o$ vs. T and $\log \bar{k}_o$ vs. T. In particular, the plots of $\bar{S}$ vs. T for β-endorphin and glucagon exhibited an overall decline as the temperature was increased, whilst the

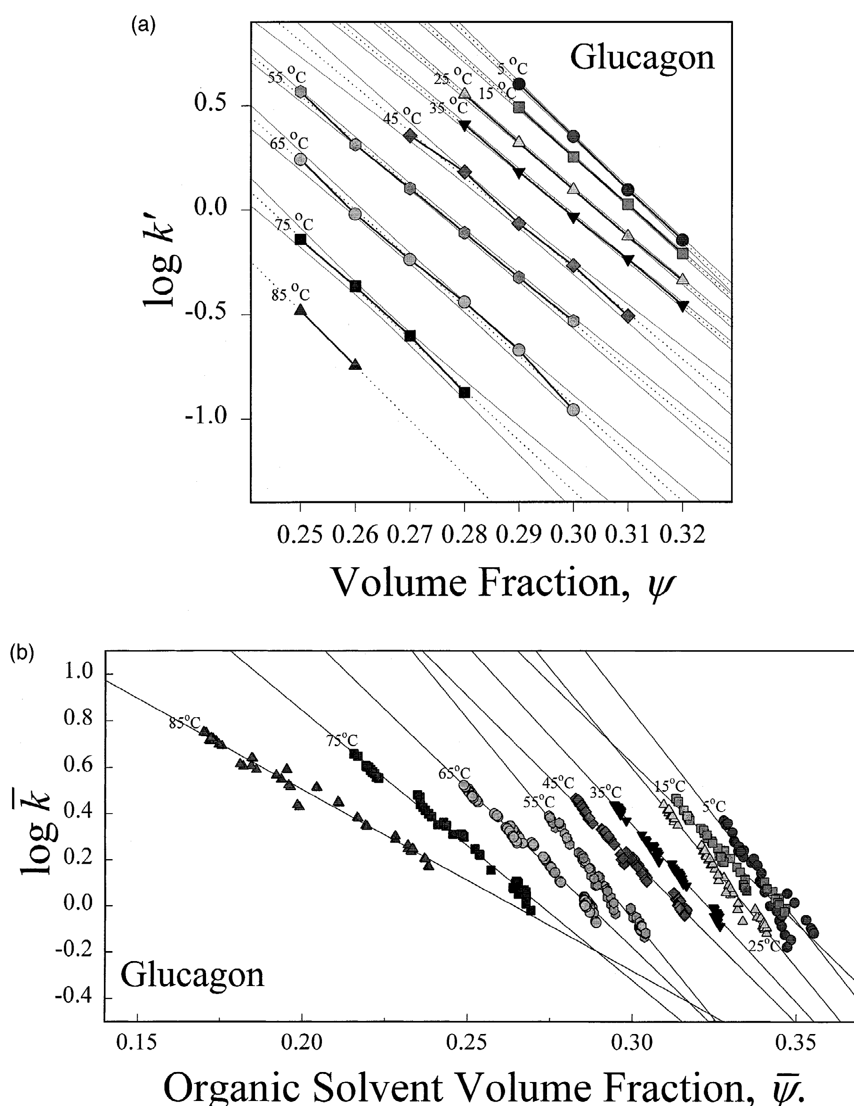

Fig. 3. (a) Plots of $\log k'$ vs. ψ for glucagon measured with a n-butylsilica reversed-phase sorbent at different temperatures from 5 to 85°C under isocratic elution conditions at a flow-rate of 1 ml/min, encompassing the range of acetonitrile concentrations from $0.25<\psi<0.32$. Also shown in this figure is the linear regression line (dotted points) and the boundary lines for confidence intervals of 95% assuming that the dependency between $\log k'$ and ψ can be described in terms of Eq. (7). (b) Plots of $\log \bar{k}$ vs $\bar{\psi}$ for glucagon measured with the same n-butylsilica reversed-phase sorbent at different temperatures from 5 to 85°C, encompassing the gradient time duration of 15, 30, 45, 60, 75, 90, 120, 150 and 180 min at a flow-rate of 1 ml/min, using linear gradient elution conditions generated from 0.1% TFA in water (buffer A) and 0.09% TFA in 65% aqueous acetonitrile (buffer B), corresponding to the notional range of acetonitrile concentrations of $0.16<\bar{\psi}<0.36$ for the elution of this polypeptide. Shown in this figure is the linear regression line, but the boundary lines for confidence intervals of 95%, assuming that the dependency between $\log \bar{k}$ and $\bar{\psi}$ can be described in terms of Eq. (11), have been omitted for reasons of clarity.

corresponding S values went through a minimum. Moreover, the extrapolated $\log \bar{k}_o$ values for β-endorphin and glucagon were essentially identical over the temperature range of 5–85°C, in contrast to the situation observed with the isocratically derived $\log k_o$ values, whilst the $\bar{S}$ values for these two polypeptides converged to a common value at temperatures above 55°C, indicative of co-elution be-

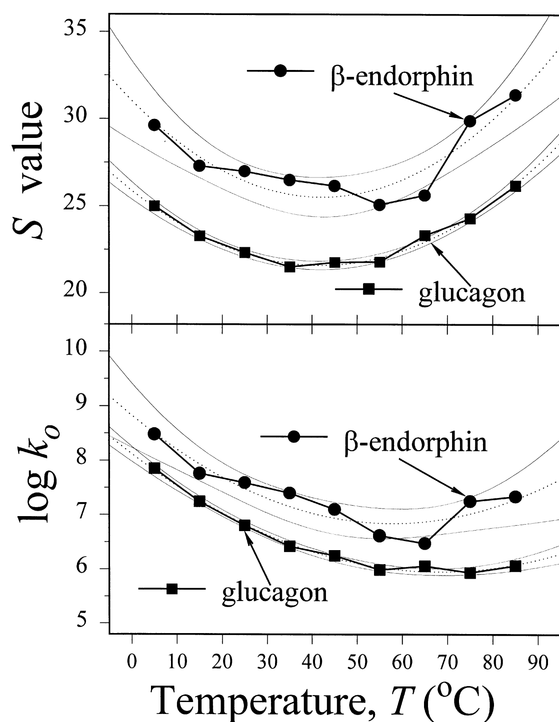

Fig. 4. Plots of the isocratic S values and $\log k_o$ values for β-endorphin and glucagon as a function of temperature, T. Also shown in this figure is the second order regression line (dotted points) and the boundary lines for confidence intervals of 95%.

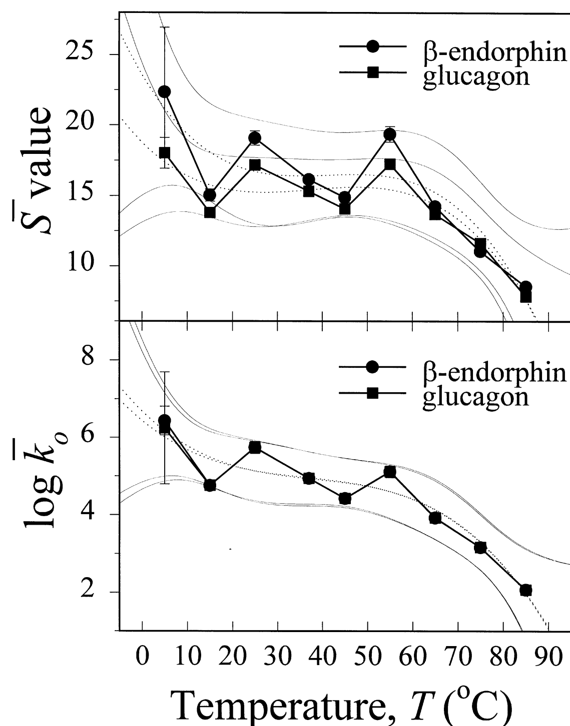

Fig. 5. Plots of the gradient-derived $\bar{S}$ values and $\log \bar{k}_o$ values for β-endorphin and glucagon as a function of temperature, T. Also shown in this figure is the third order regression line (dotted points) and the boundary lines for confidence intervals of 95%.

haviour under these conditions. These striking differences between the S and $\bar{S}$ values (or the $\log k_o$ and $\log \bar{k}_o$ values) may thus provide an explanation why gradient-derived $\bar{S}$ values of polypeptides compared to isocratic S values poorly correlate (r^2 typically ≤ 0.57 [41] for polypeptides eluted under gradient conditions) with the total surface area, ΔA_T, of a polypeptide that is accessible to a solvent probe such as a water molecule [41–43].

3.4. Dependency of log k′ and log $\bar{k}_o$ on temperature for β-endorphin and glucagon as a function of the organic solvent modifier concentration

The above results demonstrate that, in contrast to low-molecular mass analytes, substantial differences occur for these polypeptides in terms of their derived S (or $\log k_o$) values and their $\bar{S}$ (or $\log \bar{k}_o$) values. These findings are thus relevant to the derivation of the plots of $\log k'$ vs. $1/T$ and $\log \bar{k}$ vs. $1/T$ and hence to the respective free energy terms, i.e. the apparent change in enthalpy of association, ΔH^o_{assoc}, or the apparent changes in the entropy of association, ΔS^o_{assoc} (taking into account the $\log \Phi/R$ terms), when RP-HPLC data are employed in investigations to evaluate the thermodynamic basis of the polypeptide–ligate interactions. When isocratic elution systems are employed, the derivation of the relevant $\log k'$ data as the temperature, T, is varied with mobile phases of different ψ values is relatively straightforward due to the nature of the experimental design and the choice of the chromatographic conditions. As a consequence, classical Van 't Hoff plots can be readily derived from presentation of the $\log k'$ data as a function of $1/T$. In Fig. 6 is shown the plots of $\log k'$ vs. $1/T$ for β-endorphin and glucagon at $\psi = 0.27$, respectively. Also shown in Fig. 6 are the corresponding plots for the low-molecular mass compounds N-acetyl-L-α-phenylalanine ethyl ester

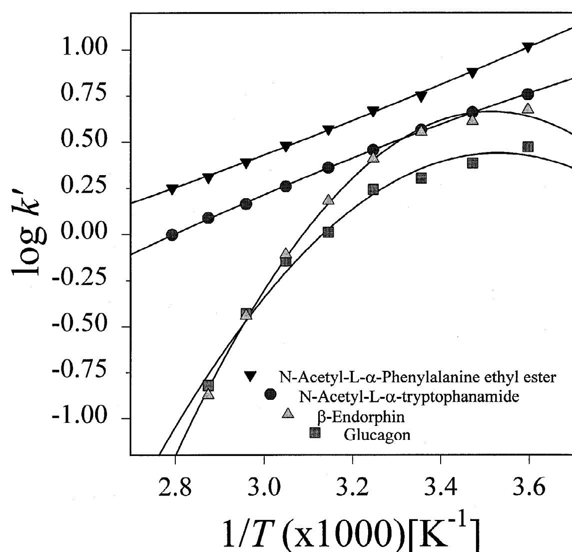

Fig. 6. Plots of $\log k'$ vs. $1/T$ for β-endorphin, glucagon, *N*-acetyl-L-α-phenyl-alanine ethyl ester and *N*-acetyl-L-α-tryptophanamide utilising retention data obtained on a *n*-butylsilica sorbent at different temperatures under isocratic elution conditions. For β-endorphin and glucagon the ψ value was 0.27, whilst for *N*-acetyl-L-α-phenylalanine ethyl ester and *N*-acetyl-L-α-tryptophanamide the value was $\psi=0.25$.

and *N*-acetyl-L-α-tryptophanamide measured at $\psi=$ 0.25 for comparison. Because of their size, these latter compounds do not undergo any conformational changes associated with secondary structure, and exhibit Van 't Hoff behaviour characteristic of systems where the heat capacity is invariant of temperature, i.e. characteristic of the isothermic interaction mode [41,44,45]. In contrast, both β-endorphin and glucagon under these isocratic RP-HPLC conditions exhibit $\log k'$ vs. $1/T$ plots illustrative of non-classical Van 't Hoff relationships involving a dependency of ΔH^{o}_{assoc} and/or ΔS^{o}_{assoc} on *T*. In subsequent papers, the origin of this non-classical Van 't Hoff behaviour with polypeptides will be elaborated [42,44–47].

Although in the case of gradient elution data the corresponding values of $\log \bar{k}_o$ and *T* can also be readily obtained, the ψ value across the chromatographic peak zone encompassing the concentration profile of the migrating polypeptide is not constant. The instantaneous value of the organic solvent modifier at the concentration mid-point of the solute's peak envelope has to be represented by a median value, $\bar{\psi}$ Accordingly, extrapolation methods must be employed with gradient elution data in order to simulate the volume fraction conditions that correspond to the ψ values of the organic solvent used in the isocratic experiments. This extrapolation can be achieved by drawing a series of intercept lines at different $\bar{\psi}$ values, typically at 0.05 intervals, with the $\log \bar{k}$ vs. $\bar{\psi}$ plots obtained at different temperatures, recording the different $\log \bar{k}$ intersection values, and then replotting these data as plots of $\log \bar{k}$ vs. $1/T$ at the different notional $\bar{\psi}$ values.

Figs. 7–9 illustrate the plots of $\log \bar{k}$ vs. *T* for *N*-acetyl-L-α-phenylalanine ethyl ester, β-endorphin and glucagon obtained in this manner. In the case of the gradient-derived results for *N*-acetyl-L-α-phenylalanine ethyl ester essentially linear $\log \bar{k}$ vs. $1/T$ plots were obtained ($r^2 \geq 0.93$), but with intercept values and slopes that progressively increase as the $\bar{\psi}$

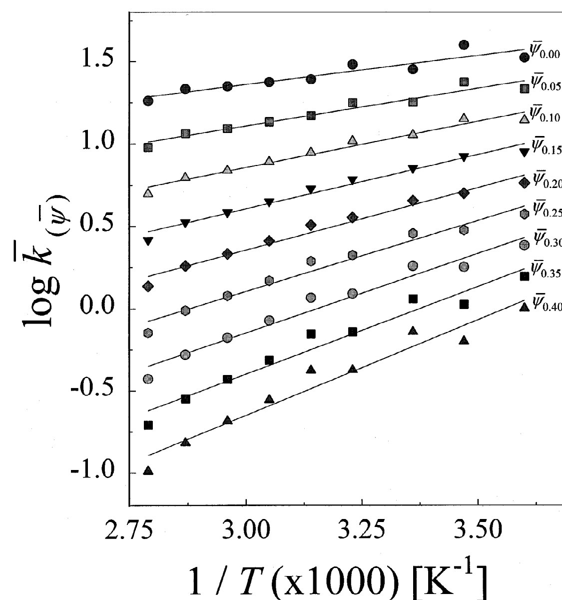

Fig. 7. Plots of $\log \bar{k}$ vs. $1/T$ for *N*-acetyl-L-α-phenyl-alanine ethyl ester utilising retention data obtained on a *n*-butylsilica sorbent at different temperatures under the gradient elution conditions. Temperatures from 5 to 85°C were employed together with gradient time duration of 15, 30, 45, 60, 75, 90, 120, 150 and 180 min at a flow-rate of 1 ml/min, using linear gradient elution conditions generated from 0.1% TFA in water (buffer A) and 0.09% TFA in 65% aqueous acetonitrile (buffer B). The data for these $\log \bar{k}$ vs. $1/T$ plots at different $\bar{\psi}$ values was derived utilising extrapolation procedures from the corresponding $\log \bar{k}$ vs. $\bar{\psi}$ plots at different values of *T*.

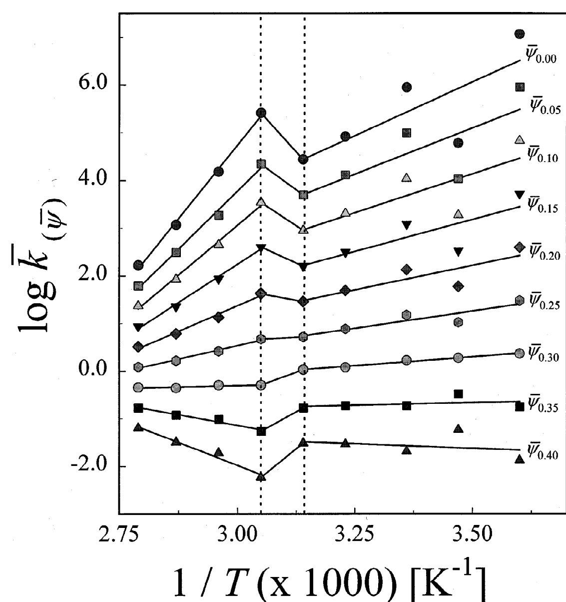

Fig. 8. Plots of $\log \bar{k}$ vs. $1/T$ for β-endorphin utilising retention data obtained on a *n*-butylsilica sorbent at different temperatures under the gradient elution conditions. Temperatures from 5 to 85°C were employed together with gradient time duration of 15, 30, 45, 60, 75, 90, 120, 150 and 180 min at a flow-rate of 1 ml/min, using linear gradient elution conditions generated from 0.1% TFA in water (buffer A) and 0.09% TFA in 65% aqueous acetonitrile (buffer B). The data for these $\log \bar{k}$ vs. $1/T$ plots at different $\bar{\psi}$ values were derived utilising extrapolation procedures from the corresponding $\log \bar{k}$ vs. $\bar{\psi}$ plots at different values of T, i.e. from the data shown in Fig. 2b.

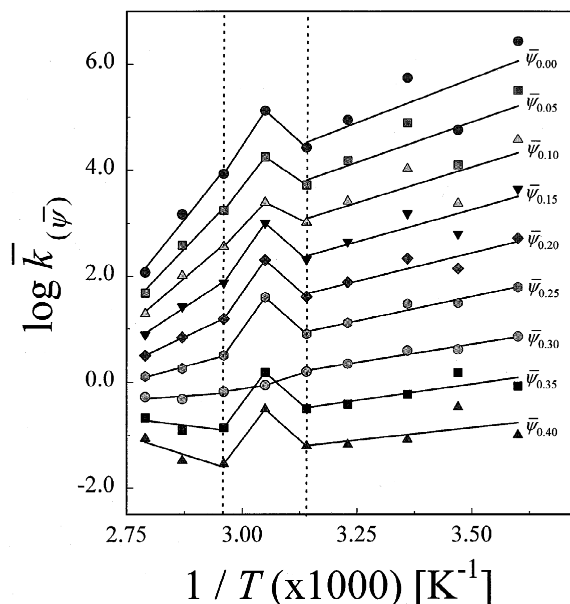

Fig. 9. Plots of $\log \bar{k}$ vs. $1/T$ for glucagon utilising retention data obtained on a *n*-butylsilica sorbent at different temperatures under the gradient elution conditions. Temperatures from 5 to 85°C were employed together with gradient time duration of 15, 30, 45, 60, 75, 90, 120, 150 and 180 min at a flow-rate of 1 ml/min, using linear gradient elution conditions generated from 0.1% TFA in water (buffer A) and 0.09% TFA in 65% aqueous acetonitrile (buffer B). The data for these $\log \bar{k}$ vs. $1/T$ plots at different $\bar{\psi}$ values were derived utilising extrapolation procedures from the corresponding $\log \bar{k}$ vs. $\bar{\psi}$ plots at different values of T, i.e. from the data shown in Fig. 3b.

values were increased. When a representative value of $\bar{\psi}$ was selected, such as $\bar{\psi}=0.25$, then the calculated $\Delta \bar{H}^{o}_{assoc}$ and $\Delta \bar{S}^{o}_{assoc}$ values, corresponding to the extrapolated intercept value as $1/T \rightarrow 0$ and the slope, were -2.96 kJ mol^{-1} and -8.5 J mol^{-1} K^{-1}, respectively. By way of comparison, the corresponding values for the apparent ΔH^{o}_{assoc} and ΔS^{o}_{assoc} determined for this solute under isocratic conditions of $\psi=0.25$ were -3.29 kJ mol^{-1} and -8.7 J mol^{-1} K^{-1}, respectively. Accordingly, these comparative data suggest that for simple (bio)solutes such as amino acid derivatives similar values for the intercept and slope at a designated ψ or $\bar{\psi}$ values may be obtained from the isocratic and gradient measurements of the dependency of the capacity factor on T, consistent with an absence in the case of low-molecular mass solutes of secondary interaction or conformational processes.

As evident from Figs. 8 and 9, much more complex plots were obtained with β-endorphin and glucagon when the gradient derived data were utilised, including discontinuity points where the slopes of these plots change value, compared to the corresponding isocratically derived plots. In this context, it can be noted that the non-monotonic deviations observed in these $\log \bar{k}$ vs. $1/T$ plots cannot be attributed to error in the measurement of the original retention or temperature data since both experimental variables showed SEM of less than 5%. These results thus highlight the limitations of assuming that the same equilibrium binding and desorption conditions prevail or are approximated in the gradient elution mode as occurs under the isocratic mode. These findings are also indicative of the participation of multiple conformational and interactive processes that occur as part of discretely different chromato-

graphic phenomena. For example, when initially introduced into the RP-HPLC column under the buffer A conditions of the gradient, both β-endorphin and glucagon will exist (on the basis of the CD results) predominantly in β-sheet and random coil conformations. From a mechanistic perspective, both polypeptides will have to undergo conformational re-organisation on contact with the non-polar ligates at the surface of the stationary phase in order to generate stabilised α-helical structures. During these transitions, different populations of contact sites will be established and then ablated as these polypeptides interact with the sorbent according to the free energy demands of these transiently solvated polypeptide–ligate complexes. Participation of silanophilic interactions can also be invoked as the polypeptide–ligate complexes undergo solvation–desolvation events as the content of the organic solvent modifier is progressively increased during the development of the gradient.

With mobile phases of low organic solvent content, the hydrocarbonaceous *n*-butyl ligates will be poorly solvated, and this ligate behaviour will provide a different binding environment for interaction with β-endorphin and glucagon than will occur with mobile phase of higher organic solvent content. Due to the heterogeneity of the sorbent surface and the close proximity (in terms of atomic distances) of the polypeptide to the sorbent, under the water-rich binding condition with buffer A of the gradient, the opportunity will also be presented for β-endorphin and glucagon to interact with transiently accessible silanol groups or other binding moieties, in addition to interacting with the non-polar ligates. Although it is generally assumed that the degree of solvation of a non-polar reversed-phase sorbent reaches a maximum value at a relatively low value of organic solvent (i.e. near 10%, v/v, for *n*-octadecyl chains immobilised onto porous silica [48,49] at room temperatures) the present findings suggest that the solvational characteristics of the sorbent are more complex at elevated temperatures particularly when gradient elution protocols are employed. Moreover, when the isocratic mode of elution is employed, these polypeptides will already exist in solvent-stabilised α-helical conformations, whilst the sorbent surface will be equivalently solvated. As a consequence, the polypeptide–ligate interaction will tend to have a less heterogeneous nature. When isocratic and gradient elution data are compared, these differences were manifested as non-equivalent dependencies of the respective capacity factors, k' or $\bar{k}$, on the experimental conditions, such as changes in temperature or the volume fraction of the organic solvent modifier. The results of our associated investigations [42,44] with all L-α- and all D-α-retro-inverso-peptide isomers and other polypeptides and small globular proteins [44–47] provide further support for these conclusions. Generally, from these and related observations on the separation and recovery of polypeptides and globular proteins from *n*-alkylsilica reversed-phase systems under gradient and isocratic elution conditions the practical codicil emerges that, from a thermodynamic and extra-thermodynamic perspective, the contributions from secondary equilibrium processes, such as conformational transitions, silanophilic interactions, or solvational changes, to the overall retention behaviour will differ in a solute-specific context under these two different elution modes.

Acknowledgements

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