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

LXVIII*. EVALUATION OF RETENTION AND BANDWIDTH RELATIONSHIPS OF PEPTIDES RELATED TO LUTEINISING HORMONE-RELEASING HORMONE AND GROWTH HORMONE-RELEASING FACTOR, SEPARATED BY GRADIENT ELUTION REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The gradient-elution behaviour of six peptides related to luteinising hormone-releasing hormone and three peptides related to growth hormone-releasing factor has been investigated using a 30-nm pore diameter *n*-butylsilica stationary phase and water–acetonitrile mobile phases containing 0.1% trifluoroacetic acid. The effect of gradient time and flow-rate on the relative retentions and bandwidths of these peptides have been assessed using quantitative expressions, derived from linear solvent strength theory and general plate height theory. Whilst the retention data for these hormonal peptides appear to be adequately described by gradient elution theory for the range of operational conditions used in this study, the experimental band broadening data are not in agreement in all cases with bandwidths predicted on the basis of plate theory derived for low-molecular-weight organic molecules. Possible causes of the "non-ideal" peak shapes observed for these peptides are discussed.

INTRODUCTION

Studies on the isolation, characterisation, and biological function of the neuroendocrine peptides of the hypothalamo-pituitary axis have expanded enormously over the past decade. This interest is largely in response to the recognition that these peptides play major roles in the regulation of a variety of endocrine and autocrine biological networks. For example, the decapeptide luteinizing hormone-releasing hormone (LHRH) (< Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is known[1,2] to function as a hypophysiotropic regulator of the secretion of the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). In addition, LHRH and its synthetic superagonists are believed to regulate sexual be-

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haviour directly at the gonadal level via control of gametogenesis and steroidogenesis. Similarly, growth hormone-releasing factor (GHRF), a 44-amino acid residue peptide isolated[3–5] from pancreatic tumors and the hypothalamus, and several synthetic analogues are known to act as key mediators of both hormonal and neural secretion of growth hormone at the anterior pituitary level mechanism. Considerable interest also centres on the synthesis of superpotent analogues and fragments of GHRF in view of their potential as modulators of lactation. Despite extensive biological investigations with these polypeptide releasing factors and their synthetic analogues, multiple factors still complicate the elucidation of their structure–activity relationships, including differences in the rate of metabolism between different analogues, differences in their distribution between different tissue types and target organs, and differences in receptor affinities. Interpretation of the physiological role of these releasing factors is made even more complicated, since products from the clearance and metabolism of these polypeptides by, for instance, the kidney, have also been implicated in the regulation of the plasma levels of the mature polypeptide releasing factor itself.

Reversed-phase high-performance liquid chromatography (RP-HPLC) has proved to be an indispensible tool in the purification and analysis of LHRH- and GHRF-related polypeptides and their synthetic analogues. Previous studies in this field have been carried out with various porous microparticulate *n*-butyl-, *n*-octyl- or *n*-octadecylsilica packings with nominal pore diameters of 6–30 nm. Furthermore, typical chromatographic isolations (*cf.*, *e.g.*, refs. 3–5) have generally employed gradient-elution conditions with methanol, acetonitrile or 2-propanol as the organic modifier. Various ionic or non-ionic additives, such as triethylammonium phosphate, have also been included in the mobile phases, both to increase solute solubilities and to suppress secondary equilibria[6,7], which these polyionic solutes can undergo in solution and at solid–liquid interfaces, such as at the reversed-phase–mobile-phase boundary.

However, it is evident from the literature that far from optimal resolution, peak shape and recovery of these polypeptides have in some cases been obtained. Recently, studies on the development of optimisation models[8–11] for the isocratic and gradient elution separation of low- and high-molecular-weight solutes by RP-HPLC have been described. In particular, concepts derived from the linear solvent strength gradient model[12] have been shown to provide a useful basis for evaluating the behaviour of polypeptides or proteins separated under regular reversed-phase conditions and to allow, in a more rational way, the alteration of chromatographic parameters to achieve optimised separation conditions. Obviously, in the absence of any rigorous models capable of predicting retention and bandwidth dependencies on experimental conditions and adequately related to the thermodynamic, kinetic, and mechanistic processes involved in the gradient separation of peptides and proteins, there has been little recourse for investigators but to change experimental conditions arbitrarily, often with only a few trial-and-error guidelines for success.

In previous studies[13,14], the application of these gradient optimisation concepts to the evaluation of various retention parameters associated with the reversed-phase gradient elution of a series of polypeptides related to human β-endorphin was illustrated. Good correlations were obtained for several small β-endorphin analogues between experimental bandwidths and peak widths predicted by analytical equations

derived from the general plate-height theory. However, in cases where chromatographic residence time effects or other surface-mediated effects resulting in the stabilisation/destabilisation of the secondary peptide structure can occur, the experimental bandwidths were found to exceed significantly the predicted values derived for a conformationally rigid molecule of identical molecular weight and hydrophobicity. In the present paper we describe similar analyses of the gradient-elution data of a set of six polypeptides related to LHRH and three polypeptides related to rat GHRF with a *n*-butylsilica as the stationary phase and a water–acetonitrile–trifluoroacetic acid (TFA) solvent system.

MATERIALS AND METHODS

Apparatus

All chromatographic measurements were obtained using a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph system, consisting of two M6000A pumps, a WISP M710B automatic injector and a M660 gradient programmer. The detector used was a M450 variable-wavelength UV monitor, operating at 215 nm and coupled to a M730 data module.

Chemicals and reagents

Acetonitrile was HPLC-grade, obtained from Millipore Corp (Lane Cove, Australia). TFA was obtained from Pierce (Rockford, IL, U.S.A.). Water was quartz-distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, U.S.A.). The LHRH-related peptides were generously obtained from Dr. Jack Johannsen (Carlbiotec, Copenhagen, Denmark) and peptides related to rat GHRF were kindly provided by Dr. G. Tregear (Howard Florey Institute, Melbourne, Australia). The amino acid compositions of the various chromatographed peptides were determined using a Durrum D500 analyser, following hydrolysis of aliquots of the recovered peptides with 6 *M* hydrochloric acid containing 0.1% phenol, for 16 h at 110°C.

Chromatographic procedures

Bulk solvents and mobile phases were filtered and degassed under vacuum. Chromatographic measurements were made at 20°C with a Bakerbond Wide-Pore C_4 Column (25 cm × 4.6 mm I.D., average particle diameter 5 μm). Linear gradient elution was carried out with water containing 0.1% TFA (solvent A) and water–acetonitrile (50:50) containing 0.1% TFA (solvent B) over gradient times varying between 20 and 120 min. Sample sizes for each polypeptide varied between 1 and 10 μg with injection volumes between 10 and 100 μl. No change in solute retention time for a defined gradient time was noted over this mass loading range. The column dead time, t_0, was taken as the retention time for sodium nitrate. The various chromatographic parameters, b, S, $\bar{k}$, k'_0, $\bar{\varphi}$, $t_{g,calc}$, PC_{exp}, PC_{calc}, D_m, b', ρ^*, $\sigma_{v,calc1}$, $\sigma_{v,calc2}$, were calculated by using the Pekinese program, written in BASIC for a Hewlett-Packard HP86B computer from input values of t_0, F, t_g, t_G, $\Delta\varphi$, x, η, MW, T, d_p, F, a', $\varphi_{v,exp}$, and L, as described previously[13,14]. The criteria for the multivariate and non-linear regression best-fit routines for the analysis of the data were based on our earlier investigations.

RESULTS AND DISCUSSION

Retention relationships

The theoretical background for the calculation of the various chromatographic parameters from experimental gradient retention data discussed in the present study has been extensively described in several previous papers[12–16]. Briefly, the capacity factors (k') of a polypeptide separated by isocratic elution in regular reversed-phase systems can be expressed as a function of organic volume fraction, φ, such that over a defined (often limited) range of $\Delta\varphi$ values

$$\log k' = \log k'_0 - S\varphi \tag{1}$$

Generally, plots of log k' *versus* φ for polypeptides, separated on *n*-alkylsilicas, are found[17–19] from experimental studies to be curved rather than linear, as predicted by eqn. 1. In these cases the tangent to the log k' *versus* φ curve can be calculated at a particular value of k', and the value of log k' at $\varphi = 0$ (log k_0) is obtained by extrapolation of the appropriate polynomial by using regression routines. Furthermore, it has been shown[12–16] that the retention time (t_g) for a polypeptide, chromatographed under gradient-elution conditions, can be related to the gradient steepness parameter, b, for linear solvent strength gradients through the expression

$$t_g = (t_0/b)\,[\log 2.3\, k'_0\, b] + t_0 + t_d \tag{2}$$

where t_0 is the column dead time, k'_0 is the capacity factor for the solute in the initial conditions of the gradient (solvent A) and is assumed to be large for peptides and proteins, and t_d is the gradient elapse time required for the change in solvent B to reach the column inlet. With most instrumental systems where $t_d \neq 0$, the total time for the change in solvent B to reach the column exit is $(t_0 + t_d) = t'_0$ and this delay will thus have a small effect on the magnitude of t_g, b and $\bar{k}$. In situations where the mobile phase induces changes in solute secondary or tertiary structure in a time dependent manner, then it will be also necessary to include additional terms in eqn. 2 to accomodate these solute–solvent systems dwell-time effects[19]. No simple approach has yet been developed for the approximation of these additional terms since precise details on rate constants of such conformational effects are generally not at hand. When solutes are chromatographed over a range of gradient times on the same column with the same mobile phase system, the gradient steepness parameter, b, can be derived by using the relationship[15]

$$b_1 = t_0 \log \beta \Big/ \left[t_{g1} - \frac{t_{g2}}{\beta} + t'_0 \frac{(t_{G1} - t_{G2})}{t_{G2}} \right] \tag{3}$$

where t_{g1} and t_{g2} are the gradient retention times of the solute at gradient times t_{G1} and t_{G2}, respectively, and the coefficient β represents the ratio of the respective gradient times ($= t_{G2}/t_{G1}$).

Evaluation of the b values from retention data determined over various gradient times allows the calculation of a range of median capacity factors, $\bar{k}$, and the

corresponding organic mole fraction, $\bar{\varphi}$, which, to a first approximation[12], can be evaluated from eqns. 4 and 5, namely

$$\bar{k} = \frac{1}{1.15b} \tag{4}$$

$$\bar{\varphi} = \left[t_{g1} - t'_0 - \left(\frac{t_0}{b}\right) \log 2 \right] \Big/ t^0_G \tag{5}$$

where $t^0_G = t_{G1}/\Delta\varphi$. The values of the parameters S and $\log k'_0$ can then be obtained from regression analysis of plots of $\log \bar{k}$ *vs.* $\bar{\varphi}$. Similar analyses allow the evaluation of these parameters for different chromatographic conditions, *i.e.* variation of the flow-rate while maintaining a constant gradient time, or same pH range but different organic solvent composition, etc. In the case of solutes chromatographed under linear solvent-strength gradient conditions at two different flow-rates, F_1 and F_2, the b value can be determined[15] from the following relationship

$$b_1 = \frac{\log (F_2/F_1)}{x_1 - x_2 (F_1/F_2)} \tag{6}$$

where

$$x_1 = \frac{t_{g1} - t_{01}}{t_{01}} \quad \text{and} \quad x_2 = \frac{t_{g2} - t_{02}}{t_{02}}$$

t_{g1} and t_{g2} are the solute retention times at F_1 and F_2 with corresponding column dead times of t_{01} and t_{02}.

Several useful predictions arise from above treatment if it is assumed that ideal reversed-phase behaviour of polypeptide solutes occurs under linear solvent strength gradient conditions. Under such elution conditions, the gradient steepness parameter, b, is constant for all solutes. Even though b is constant, under these conditions both linear and non-linear dependencies of $\log \bar{k}$ on $\bar{\varphi}$ may be found experimentally. Over limited ranges of $\Delta\varphi$ where the retention behaviour of the solutes will presumably involve only minor changes in secondary equilibria associated with surface or conformational effects, the relationships between $\log \bar{k}$ and $\bar{\varphi}$ can be approximated to linear dependencies. The participation of secondary conformational equilibria kinetics, although important, will certainly not be the sole condition for the deviation of the retention behaviour of polypeptides from this empirical generalisation. Other effects, mediated through silanophilic interactions or through the heterogeneity of the distribution of the hydrocarbonaceous ligand because of irregularities in pore geometry and surface topography to mention but two phenomena, could also lead to retention deviations. Secondly, under conditions of changing b value, *i.e.* under different t_G or F conditions, changes in bandspacing may become evident with peptide sets for which the absorption/desorption phenomena involve different mechanistic processes. It is likely although not yet proven that changes in retention processes which are mediated through different classes of binding sites on the heterogeneous stationary-phase surface or through different topographic regions of the exposed, solvated surface structure of the solute lead to different values of S for the compounds

TABLE I

STRUCTURES AND RETENTION PARAMETERS OF LHRH- AND GHRF-RELATED PEPTIDES

Peptide	*Sequence*	*MW*	$D_m \cdot 10^7$ (cm^2/s)	*S*	$\log k'_0$	$\chi 1$	$\chi 2$	$\chi 3$	$\chi 4$
LHRH-1	<EH-OCH_3	298	4.22	63.09	−0.19	0.23	−0.76	4.02	0.95
LHRH-2	<EHW	470	3.63	11.85	1.79	2.01	−0.97	13.70	16.50
LHRH-3	<EHWS	557	3.43	13.46	1.56	1.45	−0.31	9.60	13.30
LHRH-4	<EHWSY	723	3.15	14.34	2.57	3.15	−1.70	15.50	20.00
LHRH-5	GLRPG-NH_2	583	3.38	19.04	1.39	2.90	2.79	36.00	17.60
LHRH-6	<EHWSYGLRPG-NH_2	1216	2.65	18.64	3.56	6.05	0.15	44.90	35.10
GHRF-1	QQGERNQE-NH_2	985	2.83	29.27	0.85	−4.56	−2.31	−12.0	−3.3
GHRF-2	RQQGERNQE-NH_2	1141	2.70	29.36	1.05	−5.66	−3.68	−15.6	−5.3
GHRF-3	IMNRQQGERNQE-NH_2	1499	2.47	24.13	1.73	−3.64	3.92	−9.1	1.5

in question. For solute analogues where exactly the same molecular region, through specific orientation of preferred conformers, is involved in the interaction with the stationary-phase surface coincidental retention behaviour with parallelism in the log $\bar{k}$ *versus* $\bar{\varphi}$ plots over the $\Delta\varphi$ range could arise (*i.e.* if the solutes have the same S value in this extreme case the selectivity, α, becomes zero over the $\Delta\varphi$ range). When these cases are anticipated for two closely related analogues, modulation of selectivity should be attempted through manipulation of secondary solution equilibria, *e.g.* through pH effects, ion-pairing effects, solvation effects, etc. Because of the intimate relationships between S, $\bar{k}$, and b, manipulation of the gradient slope should always be attempted in several pilot experiments when the molecular homogeneity of a particular peak zone eluted under one condition is suspect (as for example illustrated by the experiments with the GHRF-related peptides discussed below).

To validate further the utility of the above predictive approach as an aid in the optimisation of the separation of neuroendocrine peptides in general and to gain further insight into the relationship between peptide structure and retention characteristics per se, gradient elution data were collected for a series of six synthetic peptides related to LHRH and three peptides related to the rat GHRF, as listed in Table I. The LHRH-related peptides ranged from 2 to 10 residues in length with corresponding molecular weights within the range of *ca.* 300–1200. The peptides LHRH-2, -3 and -4 are amino-terminal LHRH fragments known[20,21] to be generated in renal homogenates by enzymatic hydrolysis of LHRH, while peptide LHRH-5 represents a carboxy-terminal chymotryptic-like fragment of peptide LHRH-6, the naturally occurring decapeptide first isolated[22,23] from ovine hypothalami. Similarly, the peptides GHRF-1, -2 and -3 are carboxy-terminal fragments corresponding to residues 30–37, 29–37, and 26–37, respectively, of the rat hypothalamic GHRF (1–43). As part of one series of experiments, values of $\bar{k}$ and $\bar{\varphi}$ were calculated from the gradient-elution times (t_g) of these peptides according to eqns. 2–5 for gradient times of t_G = 20, 30, 40, 60, and 120 min at a fixed flow-rate of 1 ml/min. In a second series of experiments, the gradient time was held constant at 40 min, while the chromatographic separations were carried out at flow-rates of 1, 2, 3, and 4 ml/min, respectively. All samples were chromatographed with a linear gradient from water containing 0.1% TFA to water–acetonitrile containing 0.1% TFA. Fig. 1 shows the plots of log $\bar{k}$ *vs.* $\bar{\varphi}$ for the LHRH-related peptides while Fig. 2 shows the corresponding data for the GHRF-related peptides. In Figs. 1 and 2 data from both constant-flow and varied-flow experiments are included. Data for the polar substance, peptide LHRH-1, were not included in this analysis, since under all conditions this peptide exhibited very low retention times and an elution behaviour characteristic of isocratic elution development. As is evident from Figs. 1 and 2, essentially linear dependencies of log $\bar{k}$ *vs.* $\bar{\varphi}$ were observed for both sets of peptides under constant and varied flow conditions, as predicted by eqns. 2–6. The correlation coefficients for the linear regression analysis of the log $\bar{k}$ *versus* $\bar{\varphi}$ data were between 0.98 and 0.99 for the various LHRH-related peptides and between 0.92 and 0.97 for the three GHRF fragments.

Changes in selectivity within each set of peptides over the experimental range of b values are clearly reflected in the different slopes or S values (Table I). Also listed in Table I are the extrapolated log $\bar{k}$ values at $\bar{\varphi}$ = 0, *i.e.* the log k'_0 values, and the relative hydrophobicities of each peptide, calculated as the summated hy-

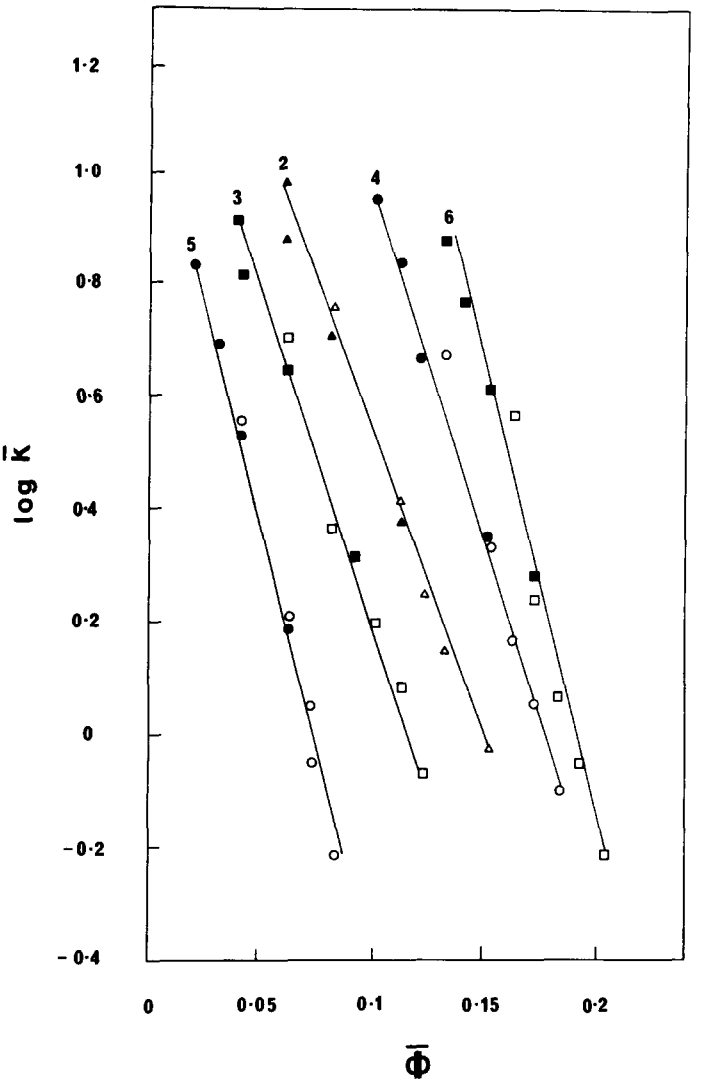

Fig. 1. Plots of log $\bar{k}$ *versus* $\bar{\varphi}$, based on gradient elution chromatography of LHRH-related peptides (LHRH-2–LHRH-6). The plots were derived from best-fit analysis to the data points shown, where t_G = 20, 30, 40, 60, and 120 min and F = 1 ml/min (open points), and F = 1, 2, 3, and 4 ml/min and t_G = 40 min (filled points). Other chromatographic conditions are given in Materials and methods. See Table I for the peptide structures and for the derived S and $\log k'_0$ values.

drophobic coefficients of individual amino acids[24–27] present in the linear sequences. In two recent studies[13,15] the relationship between solute S values and solute molecular weight has been discussed. In both of these investigations empirical dependencies of the form $S = a\,(\mathrm{MW})^b$ have been derived, but in both studies the values of the coefficients a and b were clearly dependent on the set of peptides or proteins under consideration. When data for the S values and MW for the current family of LHRH-related peptides were fitted by similar non-linear regression analyses, the fol-

lowing "best-fit" relationship was obtained

$$S = 1.42\ MW^{0.36} \quad (7)$$

with a correlation coefficient (r^2) of 0.42. Clearly, changes in S values must be related to differences in the magnitude of the contact area and in the number of interaction sites established between the solute and the stationary phase during the adsorption process rather than to an increase in molecular mass *per se*. Although an increase in molecular weight through, for *e.g.*, peptide chain elongation may result in an increase in the S value and also an increase in the Stokes radius of the solute, as evidenced by an increase in hydrodynamic volume, depending on the nature and distribution of the additional amino acid residues, it does not necessarily follow that simple relationships between molecular mass and S values will occur. Implicit in the molec-

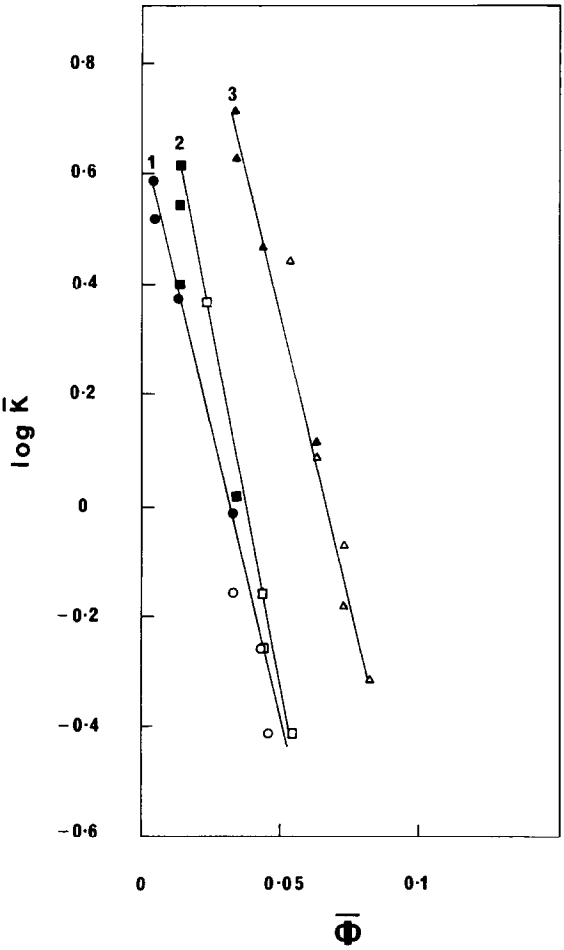

Fig. 2. Plots of log $\bar{k}$ *versus* $\bar{\varphi}$ based on gradient elution chromatography of rat GHRF-related peptides (GHRF-1–GHRF-3) under the experimental conditions described in the legend to Fig. 1. See Table I for peptide structures and calculated S and $\log k'_0$ values.

ular cryptic features of the primary sequence of any polypeptide is the potential for secondary and tertiary structure development under a particular set of conditions. The mechanistic nature of the solute interaction with the stationary phase will thus be closely related to the polarity and disposition of the surface residues of the peptidic solute and whether these residues can adopt a preferred conformation within the microenvironment of the solvated hydrocarbonaceous ligand chains. Hence, if the peptide assumes any degree of preferred folding, no simple relationship can *a priori* exist between the S value and the corresponding summated hydrophobicity (χ) values of all the amino acid residues, read as the total linear sequence. Similar requirements would obviously apply to the relationship between the solute S value and molecular weight. These conclusions are reinforced by the relatively low correlation coefficients found when the S values and the χ values of the various peptides were fitted to linear, exponential, logarithmic, and power functions of the forms $S = a + b\chi$, $S = a\,e^{b\chi}$, $S = a + b \log \chi$, and $S = a\chi^b$ respectively, using a non-linear least-squares curve-fitting program. The coefficients of correlation for these comparative analyses are given in Table II. Generally, poorer correlations were observed for S *versus* χ_1, (Rekker and de Kart scale[24]), χ_2 (Su *et al.* scale[25]) and χ_4 (Meek and Rossetti scale[27]) which confirms that the linear summated hydrophobicity values, derived with ion-pairing systems different from the TFA–water–acetonitrile system used in the present study, bear only a limited relation to the solvated solute structure which ultimately binds to the stationary phase. The higher correlation coefficients of $r^2 = 0.82$–0.86 for S *vs.* χ_3[26] (which takes into account peptide–TFA-mediated stationary-phase interactions) serves to illustrate how apparent hydrophobicity contributions of each amino acid can more closely approximate the surface polarity of the interacting solute when a data base, derived for the same elution system, is employed.

Similar curve-fitting procedures were carried out to evaluate possible dependencies of log k_0 values on MW and χ_i for the LHRH peptides. The results are also

TABLE II

CORRELATION, r^2, OF THE SLOPE PARAMETER, S, AND THE LOGARITHMIC CAPACITY FACTOR IN NEAT WATER, log k'_0, WITH MW, AND SUMMATED HYDROPHOBICITY COEFFICIENTS FOR THE LHRH-RELATED PEPTIDES

Correlation coefficients (r^2) were determined for the relationships $y = ax + b$ (LIN), $y = ae^{bx}$ (EXP), $y = a + b \log x$ (LOG), and $y = ax^b$ (PWR), where $y = \log k'_0$ or S, and x = MW, χ_1, χ_2, χ_3 or χ_4.

	LIN	*EXP*	*LOG*	*PWR*
S versus				
MW	0.37	0.38	0.39	0.41
χ_1	0.48	0.48	0.52	0.52
χ_2	0.56	0.56	–	–
χ_3	0.86	0.83	0.84	0.82
χ_4	0.36	0.36	0.37	0.37
log k'_0 *versus*				
MW	0.87	0.78	0.85	0.77
χ_1	0.80	0.71	0.71	0.64
χ_2	0.12	0.19	–	–
χ_3	0.27	0.18	0.24	0.16
χ_4	0.86	0.78	0.85	0.78

shown in Table II. Since the log k'_0 value is a measure of the free-energy changes associated with binding of the solute to the stationary phase (including the relative solubility of the solute in the hydrocarbonaceous phase) under the initial gradient (solvent A) conditions as solute hydrophobicities increase, then increases in log k'_0 could also be intuitively anticipated. A previous study[13] from this laboratory has indicated that the relationship between log k'_0 and MW or log k'_0 and relative summated residue hydrophobicities cannot be readily described in terms of first-order dependencies, such as linear or simple power functions. The present results can be compared with similar observations, obtained previously with 29 human β-endorphin-related peptides, where again relatively poor correlations between extrapolated log k'_0 and χ-coefficient values were evident. Even taking into account the limited data base of the present study, it appears reasonable to reach a similar conclusion for both the LHRH and GHRF peptides. As a consequence, empirical equations, which attempt to link in physical terms the relationships between the retention parameters, such as S or log k'_0 with MW or χ_i, would appear at this stage to have only limited predictive value except possibly as a guide for discriminating unrelated "test" peptides and proteins of widely differing MW and structure, where the appropriate retention coefficients cannot be derived relatively easily by other chromatographic means.

There are several additional lines of evidence which suggest that the S value is not directly dependent on MW or the average summated hydrophobicity, χ, of a polypeptide or protein. Firstly, the S values of a particular polypeptide or protein eluted from non-porous supports are approximately ten times smaller than the S value observed with a comparable porous support material[28]. Secondly, reversed phase, hydrophobic interaction and ion-exchange HPLC of proteins all exhibit a pronounced dependency on solute orientation to the surface. An interesting example of this preference is the recent study[29] on the retention behaviour of various lysozymes under hydrophobic interaction chromatography conditions. Thirdly, preliminary results[30] have suggested that the S value of a protein in the denatured state is larger than the same protein in the native state eluted under similar reversed-phase chromatographic conditions[13,14,17]. Fourthly, experimental studies have shown that solutes with similar molecular weights can have widely different S values. It is not uncommon to find small peptides with large S values; invariably these peptides have stabilised hydrophobic regions. Conversely, larger, more polar peptides can have smaller S values. The magnitude of the S value of a polypeptide or protein is very dependent on the nature of the organic modifier. All these lines of evidence suggest that the S value directly relates to the hydrophobic contact area established by the solute at the stationary phase surface rather than the MW *per se*. Similarly, the S value does not appear to depend directly on the average hydrophobicity of the polypeptide or protein. Conceptually this is not surprising since it is the asymmetric distribution of the amino acid residues in three dimensional space which predicates the biological and structural uniqueness of the solute. The orientation and availability of the amino acid side chains to the stationary phase surface will thus reflect this asymmetry. Solutes which have several hydrophobic regions appropriately juxtapositioned to the contoured surface of the hydrocarbonaceous ligands will be more vulnerable to multi-site binding than solutes with only a single region or more distal regions incapable of simultaneous interaction with the non-polar stationary phase

surface. This propensity may account for the aberrant binding behaviour seen with proteins with mesoporous *n*-alkylsilicas whereby poor recovery is obtained. In these cases the solute may simply be encapsulated by many ligands due to the geometry of the pore structure.

Comparison of the data for the GHRF-related peptides with the data for the LHRH-related peptides reveals a further important observation. Inspection of the log $\bar{k}$ *versus* $\bar{\varphi}$ dependencies clearly shows that the S values for the GHRF-related peptides are larger than the LHRH-related peptides. However, the corresponding log

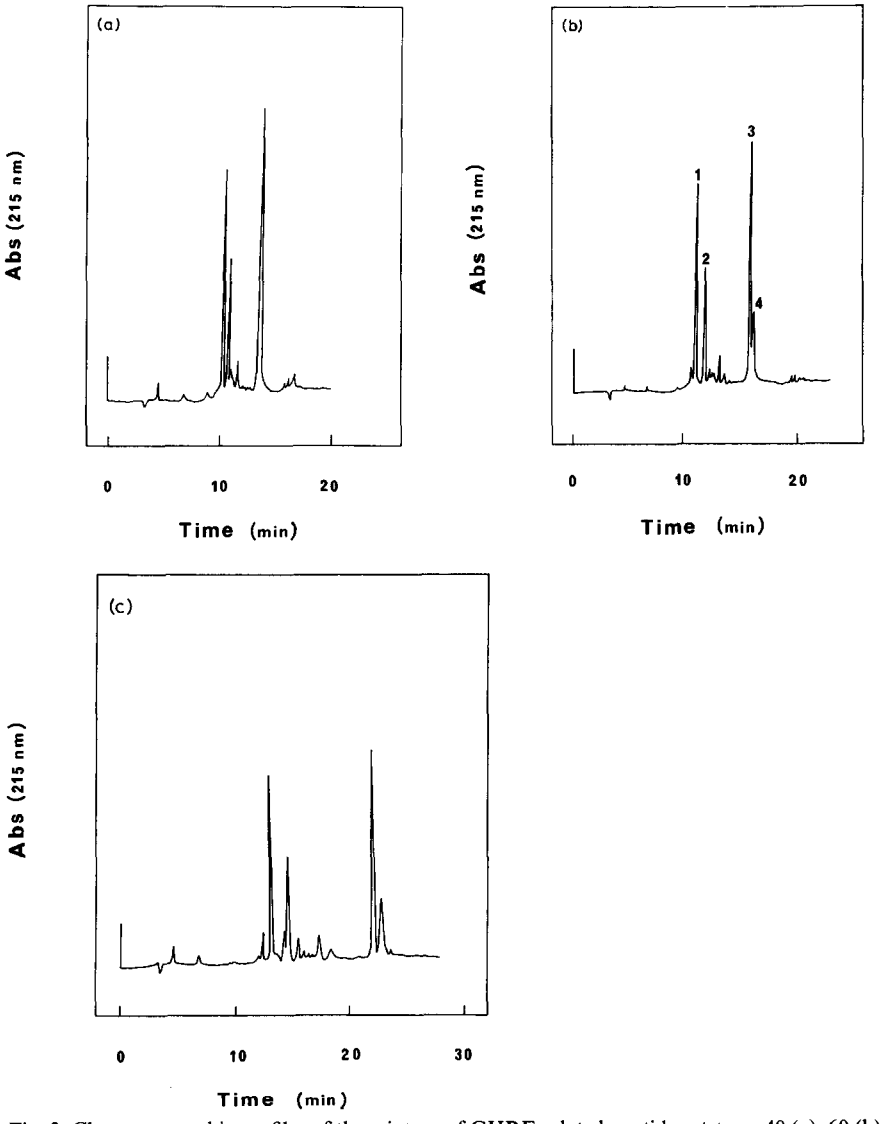

Fig. 3. Chromatographic profiles of the mixture of GHRF-related peptides at t_G = 40 (a), 60 (b), and 120 min (c) and F = 1 ml/min, illustrating the resolution of a fourth, contaminating peptide from GHRF at the longer gradient times. Other experimental conditions are given in Materials and methods.

k'_0 values for the GHRF-related peptides are uniformly smaller than for the LHRH-related peptides. This behaviour is entirely in accord with the predictions made[11] on the basis of our co-operative retention model for peptide retention to *n*-alkylsilicas. These data for the LHRH- and GHRF-related polypeptides thus extend our earlier observations, which suggested that some peptides families can be characterised in low-pH elution systems by large S values but relatively small log k'_0 values, whilst other families (possibly the majority of small globular proteins and linear polypeptides) fall into two classes: (1) low S values and low log k'_0 values, and (2) large S values and large log k'_0 values. To date, examples of the remaining class, namely, low S values but high log k'_0 values have yet to be accurately defined, but self-aggregating membrane proteins and hydrophobic proteins, such as inhibin[31–33], may represent examples of this class. In fact, the ability to describe retention behaviour of polypeptides and proteins, separated on hydrophobic columns, in terms of a multidimensional matrix, involving S values, log k'_0 values, and appropriate surface hydrophobicity parameters, clearly is analogous to other forms of selectivity maps, based on retention indices.

The value of utilising changes in gradient time to influence bandspacing is demonstrated in the purification of a synthetic fragment [GHRF-(26–37)] of rat GHRF from a partially fractionated preparation, following solid-phase synthesis. It was evident from the chromatographic and compositional results obtained following separation of the mixture with a 60-min gradient that the eluted zone corresponding to peptide GHRF-(26–37) was contaminated with a second component of similar selectivity. By taking advantage in a rational way of the different slope dependencies for these two peptides it was possible with an increase in the gradient time by a factor of two whilst maintaining F constant, *i.e.* by varying b, to separate the desired product completely from this contaminant. Subsequently, structural analysis confirmed that the contaminant was a deamidated form of peptide GHRF-(26–37). The modulation of selectivity by changes in b value or by variation in $\bar{k}$ values has considerable potential for the resolution of microheterogeneous peptides in general (see also ref. 34) and has been exploited[35] in this laboratory in other studies on protein hormones.

It can be concluded from the above data, as well as from previous data[13–15] for other polypeptides, that the theoretical treatment based on linear-solvent-strength concepts used to derive log $\bar{k}$ *versus* $\bar{\varphi}$ relationships, provides a useful approximation to the observed results of gradient elution. However, for such non-mechanistic models to have wide utility they should also adequately describe and differentiate different bandbroadening phenomena. These aspects can be evaluated either from comparisons of the relationships between peak capacity (PC) and separation variables or from comparisons of experimental bandwidths with the bandwidths calculated on the basis that zone dispersion for the peptide is described by the plate theory originally derived for low-molecular-weight solutes over a wide range of gradient conditions.

Bandwidth relationships

The peak capacity for a chromatographic separation of gradient time t_G, and average resolution equal to 1 for all peptides pairs can be given by

$$PC = t_G F/4\sigma_v \tag{8}$$

where σ_v is the bandwidth, measured in volume units (1 standard deviation). Furthermore, the relationship between σ_v and $\bar{k}$ for linear solvent systems can be expressed as

$$\sigma_{v,calc1} = [(\bar{k}/2) + 1]\, G\, V_m\, N^{-0.5} \tag{9}$$

or alternatively as

$$\sigma_{v,calc2} = t_G\, F(1 + 1/\bar{k})/(\ln 10)\, N^{0.5} S \Delta\varphi \tag{10}$$

where G, the band compression factor, which arises from the increase in solvent strength across the solute zone as the gradient develops along the column, is given by the expression[12]

$$G^2 = [1 + 2.3b + 1/3(2.3b)^2]/(1 + 2.3b) \tag{11}$$

and V_m is the column void volume (equal to $t_0 F$). Stadalius *et al.*[36] have introduced an empirical factor J to accommodate the general observation that experimental values of σ_v are larger than values predicted by eqn. 10 for steep gradients. This factor J (equal to $\sigma_{v,exp}/\sigma_{v,calc}$) is a function of $\bar{k}$. For small values of the gradient steepness parameter, b, the factor J is near to unity but approaches a limit of *ca.* 1.8 for $b > 2$. Although the basic phenomenon which leads to the J effect is not yet understood, it is important to recognise that the J effect should apply equally to all peptides as $\bar{k}$ is changed over the same range, *i.e.* relative changes in the factor J are proportional to $\Delta\bar{k}$ and at a particular b value a common value for the factor J will exist. For shallow gradients where $J \approx 1$ divergences in bandwidth must be attributed to other phenomena.

Under typical flow-rate conditions employed for the separation of polypeptides on reversed phases, the reduced velocites are often greater than 100. When such linear velocity conditions apply, the plate number, N, can be approximated by

$$N = \frac{D_m\, t_0}{C\, d_p^2} \tag{12}$$

where d_p is the mean particle diameter. C is the Knox equation parameter which accounts for the resistance to mass transfer at the stationary phase surface and is determined by

$$C = \frac{[(1 - x + \bar{k})/(1 + \bar{k})]^2}{15\,\rho a' + 15\,\rho b' k' - 19.2\rho\, x} \tag{13}$$

where x is the interstitial column volume fraction, taken here to be 0.67; a' is equal to 1.1; b' is the surface diffusion parameter, and ρ is the restricted diffusion parameter. For calculations used in the present study the diffusion coefficient of the solute in the mobile phase (D_m) was expressed in terms of the solute molecular weight and was evaluated[14] from the relationship

$$D_m = \frac{8.34 \cdot 10^{-10}\, T}{\eta\, MW^{0.33}} \tag{14}$$

where T is the absolute temperature (Kelvin) and η is the eluent viscosity.

Current non-mechanistic hypotheses describing the kinetic and thermodynamic aspects of the reversed-phase HPLC separation of peptides and proteins generally assume that the solute migrates as a unique, conformationally rigid molecule. As discussed above, the retention behaviour of some polypeptides can in special cases be predicted with reasonable accuracy on the basis of this assumption. While it is generally accepted that peptides and proteins can explore in solution a variety of conformational options, the assumption that these processes are extremely rapid compared to the chromatographic separation time has been shown to be untenable for a number of polypeptides and proteins (*cf.*, *e.g.*, refs. 18 and 37). If for the purposes of discussion, it is argued that the dynamic behaviour of the polypeptide can be represented by the migration of a single, conformationally invariant species, then it would be anticipated from currently theoretical considerations that 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, *i.e.* over the range $1 < \bar{k} < 10$. If, however, the molecular dimensions and secondary structure of the peptide are changing during its passage along the column in a relatively slow, time-dependent manner, corresponding changes in the diffusional and interactive properties of the solute should lead to differential zone migration. These increases or decreases in diffusional properties, manifested as changes in b', ρ, D_m, D_s, d_s, etc, will ultimately be revealed as experimentally observed bandwidths and peak moments that deviate from the values predicted by current general plate theory, *i.e.* from peak-widths predicted by either eqns. 9 or 10.

On the basis of dependencies inherent to eqns. 9 and 10 and using experimental chromatographic retention data, the "ideal" bandwidth values for each peptide solute were thus calculated and compared with the experimental bandwidths as a function of the gradient steepness parameter, b. The values of $\sigma_{v,calc}$ were derived from numerical substitution of eqns. 3, 4, 6, 9, 11, 12, and 13, using input values of $\bar{k}$, γ, etc. Fig. 4a shows the plot of $\sigma_{v,exp}/\sigma_{v,calc1}$ for the LHRH-related peptide series as a function of $1/b$ for chromatographic experiments carried out a constant flow-rate of 1 ml/min. Similarly, plots of equivalent shape were also obtained by using eqn. 10 for the calculation of $\sigma_{v,calc}$. The average standard deviation for the data points from these "best fit" curves was *ca.* 15%. With the LHRH-related peptides LHRH-2, -3, -4 and -6, the σ_v ratio approached a minimum value as the magnitude of $1/b$ increased. Furthermore, when allowances are made for the experimental errors involved in the measurement of $\sigma_{v,exp}$ and the errors inherent to the derivations of $\sigma_{v,calc}$ from eqn. 9, the ratio of $\sigma_{v,exp}/\sigma_{v,calc}$ for these four LHRH-related peptides does, in fact, approach a limit close in value to unity. However, the experimental σ_v to calculated σ_v bandwidth ratio for peptide LHRH-5 exhibits a parabolic dependence on $1/b$, reaching a maximum value at $b \simeq 0.5$. In addition, the experimental bandwidth for peptide LHRH-5 in the constant flow-rate experiments is significantly larger than that predicted by eqn. 9 for a peptide of MW = 583. Similar, apparently anomalous, results have been also obtained previously[13] with various β-endorphin-related peptides. The present observations with peptide LHRH-5 provide a further example of a peptide the bandwidth behaviour of which, under a given set of gradient chromatographic conditions, appears not to be fully consistent with the simple kinetic model underlying eqns. 9 and 10. At this stage of development, the influence of pore structure heterogeneity on polypeptide bandbroadening has not been determined in

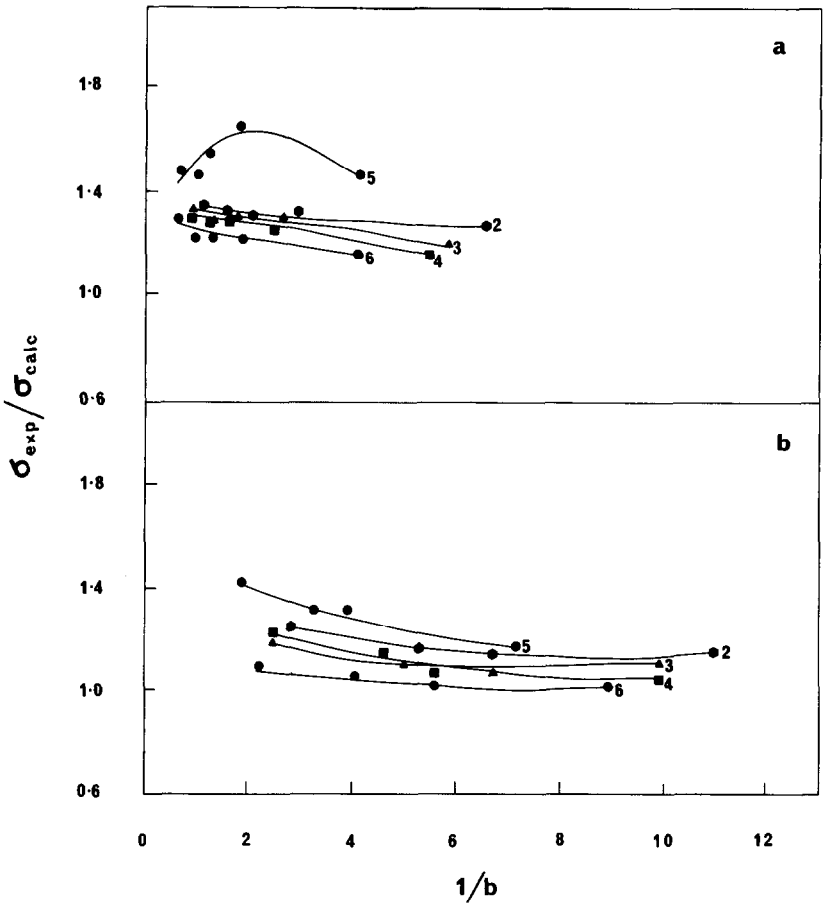

Fig. 4. Plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for the LHRH-related peptides LHRH-2–LHRH-6. (a) The dependencies shown correspond to data acquired under conditions of different gradient times, as in Fig. 1, at F = 1 ml/min. (b) Data from the experiments at constant gradient time of 40 min with F = 1–4 ml/min were used. In both cases $\sigma_{v,calc}$ was evaluated by using the equation $\sigma_{v,calc} = V_m N^{-0.5} (k/2 + 1) G$ (eqn. 9).

detail. Whether or not the contour surface of the stationary phase pores involves micro-irregularities which will lead to diffuse kinetic behaviour, the issue remains that the solutes themselves act as reporter probes of these phenomena. With a family of related peptides, subtle divergences in bandbroadening thus take on important significance since they can reflect more adequately conformational, orientational or silanophilic differences associated with the interactive process. Fig. 4b shows the plot of $\sigma_{v,exp}/\sigma_{v,calc1}$ *versus* $1/b$ for the LHRH-related peptides where t_G is maintained constant and the flow-rate is varied between 1 and 4 ml/min. Once again, it can be seen that the bandwidth ratio ($\sigma_{v,exp}$ *versus* $\sigma_{v,calc1}$) approaches a minimum value as b decreases. In this case, larger b values are associated with lower flow-rates. As anticipated larger divergences of $\sigma_{v,exp}$ from $\sigma_{v,calc}$ were observed at the lower flow-rates. This behaviour is consistent with the general observation of greater de-

viations in bandwidth for solutes eluted at large b values. The bandwidth behaviour of peptide LHRH-5 can be seen once again to deviate from the predicted values more than that of the remaining peptides, *i.e.* the order based on D_m or MW considerations should have been $\sigma_{v,calc}$ 2 < 3 ≃ 5 < 4 < 6. Nevertheless, peptide LHRH-5 also appears to follow a similar dependence on $1/b$ in reaching a minimum at higher flow-rates.

The conclusion can be drawn from these kinetic and retention data that the conformation of the binding regions of peptides LHRH-1, -2, -4 and -6 may be similar at the stationary-phase surface. This situation would require peptide LHRH-6 to exhibit behaviour of two secondary structural regions, composed of residues <E′ → Y^5 and G^6 → G^{10}, *i.e.* for the molecule to be folded with a β-turn at G^6-L^7, such that the residue G^6 is exposed to the solvent and residue R^8 is seques-

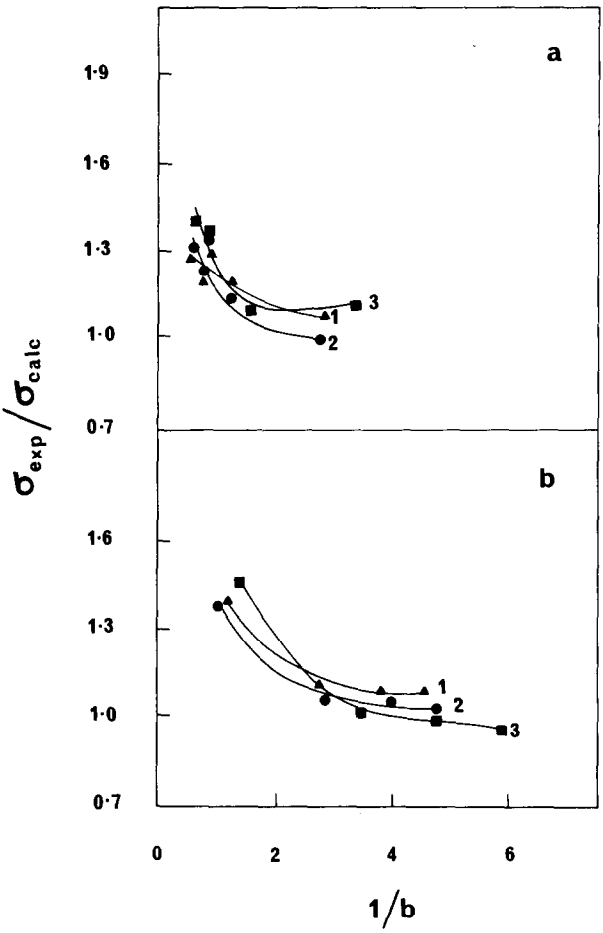

Fig. 5. Plots of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for the GHRF-related peptides GHRF-1–GHRF-3 for varied t_G and constant F (a), and constant t_G and varied F (b), obtained by using the conditions described in the legend to Fig. 4.

tered within the secondary structure, at least as far as the disposition of the folded peptide-chain N–H and backbone groups are concerned. This conclusion is in accord with recent high-field NMR studies and minimum-energy calculations[38,39] on LHRH analogues, which suggest that there is a preferred average conformation of the molecule in solution, involving an intrachain hydrogen bond-stabilised structure between $R^8NH_2 \rightarrow Y^5CO$ and $Y^5 \rightarrow R^8$, flanking a 6–7 β-turn. As a consequence, the orientation of the peptide LHRH-6 at the stationary phase surface may possibly involve a preferred contact between residues < EHWSY.... and the hydrophobic *n*-butyl ligand. This conclusion is also consistent with the pattern of results observed with log $\bar{k}$ *versus* $\bar{\varphi}$ plots, where the chromatographic behaviour of peptide LHRH-4 is more similar to peptide LHRH-6 in terms of relative hydrophobicity/retention dependency than is peptide LHRH-5.

Fig. 5a and b show the plot of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for the GHRF-related peptides, at constant flow and varied flow-rate, respectively. As can be seen from Fig. 5a, the experimental to calculated bandwidth ratios again approach a minimum value as the magnitude of $1/b$ increases. At larger b values or shorter gradient times, $\sigma_{v,exp}$ exceeds $\sigma_{v,calc}$ by up to 50% in the case of peptide GHRF-3. With weakly retained peptides, like these GHRF analogues, this behaviour may be a consequence of different patterns of gradient band compression, *i.e.* with very steep gradients and short gradient times the solutes are not exposed to comparable linear gradient elution development between 0% and 50% organic modifier, but rather pseudo-stepwise elution conditions. With very short gradient times, an increasingly significant proportion of the total elution time will be accounted for by the gradient elapse time and this will lead to increasing error in the measurement of the true $\bar{k}$. As a result, the input value of the band compression factor (G value), estimated according to the experimental $\bar{k}$ or b value, will represent an overestimation of the actual effect of gradient slope. Since compression of the band can only take place after the solute zone begins to migrate, any effect which tends to delay the migration of the zone or under estimation of b will result in apparent error in the calculation of the peak bandbroadening. In the extreme case of gradients with very large b values, the peptides will migrate in an essentially isocratic size exclusion manner with little or no resolution. Why peptide zones in these circumstances are experimentally larger than predicted is not immediately evident and the underlying phenomenon clearly deserves further investigation. Fig. 5b shows the dependence of $\sigma_{v,exp}/\sigma_{v,calc}$ *versus* $1/b$ for these GHRF-related peptides with a constant gradient time and varied flow-rate. As observed for the LHRH peptides, the largest divergence of $\sigma_{v,exp}$ from $\sigma_{v,calc}$ occurred at the lowest flow-rate of 1 ml/min, whilst the ratio approached a minimum at lower b values or higher flow-rates.

Further insight into these bandbroadening relationships can also be gained from the analysis of the experimental and the calculated peak capacity data. It has been previously demonstrated[14,40] that experimental retention data can be used to calculate a predicted peak capacity according to the relationship

$$PC = \left(\frac{\ln 10}{4}\right) \frac{S\Delta\varphi k^{0.25}}{F\, d_p} \left(\frac{D_m\, t_0}{C}\right)^{0.5} \tag{15}$$

Hence, for peptide solutes separated under defined gradient conditions, linear de-

pendencies are expected between peak capacity and $\bar{k}^{0.25}/C^{0.5}$, provided a regular reversed-phase retention mechanism is operating and conformational reorientation of the solute does not occur with half-lives similar to the time of zone transport in the chromatographic separation. Eqn. 15 also predicts that PC should increase with increased S value for a particular peptide.

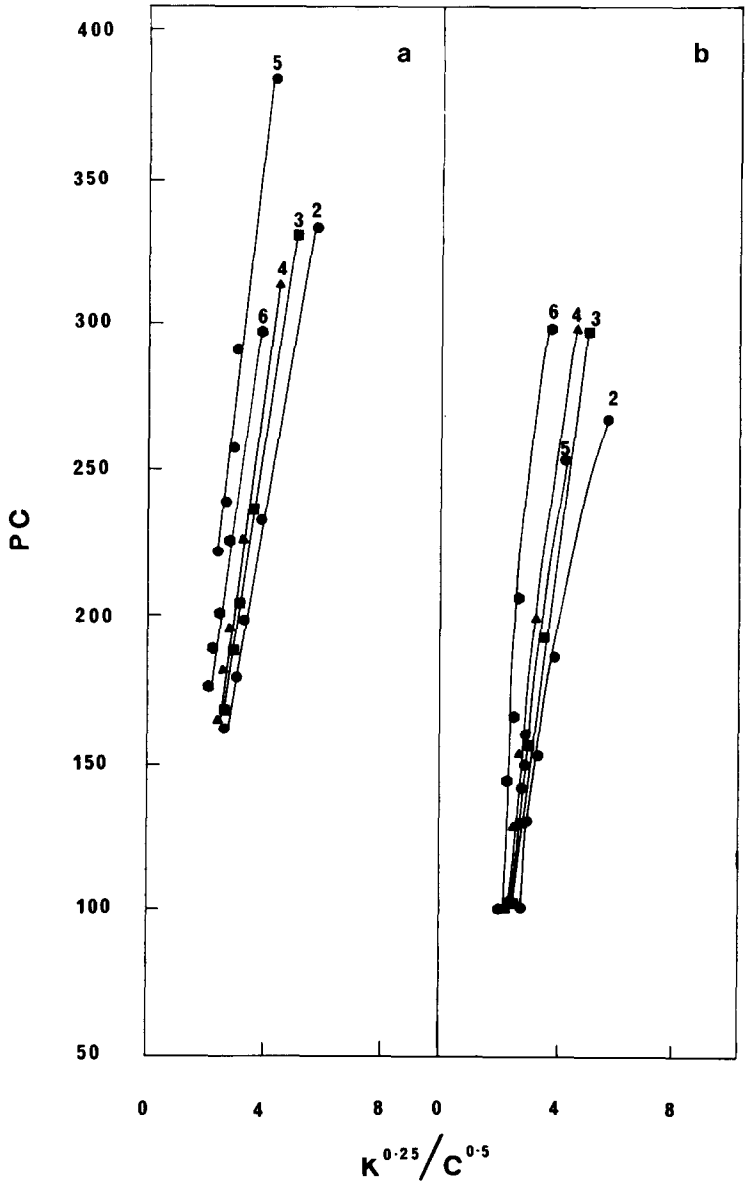

Fig. 6. Plots of peak capacity (PC) *versus* $\bar{k}^{0.25}/C^{0.5}$ for the LHRH-related peptides LHRH-2–LHRH-6. The data correspond to calculated values obtained for the varied t_G experiments (T_G = 20, 30, 40, 60, and 120 min) and F = 1 ml/min. (a) PC value determined from the equation PC = [1.15 $S\Delta\varphi$ (t_0 D_m)$^{0.5}$ $\bar{k}^{1/4}$]/2 d_p $C^{0.5}$ (eqn. 15). (b) PC value calculated from the equation PC = $t_G/4\sigma_{t,exp}$ (eqn. 8).

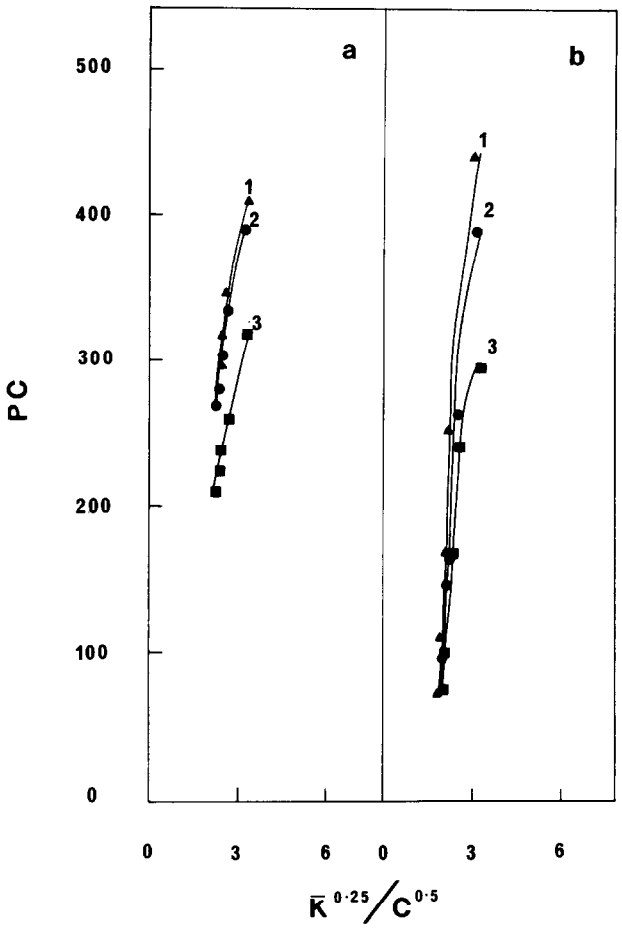

Fig. 7. Plots of peak capacity (PC) *versus* $\bar{k}^{0.25}/C^{0.5}$ for the GHRF-related peptides GHRF-1–GHRF-3. The experimental conditions are described in the legend for Fig. 6.

Fig. 6 shows the plot of PC *versus* $\bar{k}^{0.25}/C^{0.5}$ for the LHRH-related peptides with varied t_G but at a constant flow-rate of 1 ml/min. In Fig. 6a, PC_{calc} is calculated according to eqn. 15, whilst in Fig. 6b the experimentally observed peak capacity, PC_{exp}, as determined by eqn. 8 was used. In both cases, essentially linear plots of PC *versus* $\bar{k}^{0.25}/C^{0.5}$ were obtained. However, while the magnitude of PC_{exp} for peptides LHRH-2, -3, -4 and -6 were comparable to the respective values of PC_{calc}, the experimental peak capacity for peptide LHRH-5 was significantly lower than the peak capacity predicted by eqn. 15. As is evident from the σ_v data discussed above, the observed peak width for peptide LHRH-5 under the different gradient conditions was larger than expected on the basis of "ideal" kinetic behaviour. Whether this difference between the $\sigma_{v,calc}$ and the $\sigma_{v,exp}$ values over typical gradient conditions represents an overestimation by eqn. 14 of the D_m value for peptide LHRH-5, or for that matter an underestimation of the D_m values of the other peptides, remains to be established. While the kinetic behaviour, including the relative diffusional character-

istics, of peptides LHRH-2, -3, -4 and -6 appear to be well approximated by eqns. 12–14, the magnitude of the apparent brandbroadening behaviour for peptide LHRH-5 appears to be much less precisely estimated. Whatever is the origin of these differences in σ_v it can be concluded that significant differences exist in the value of the surface diffusion and the restricted diffusion term for this peptide, compared to, *e.g.*, peptide LHRH-6. Studies currently underway in this laboratory should resolve further the origin of these apparent diffusional differences with this peptide series and other series of hormonal peptides.

The plots of PC_{calc} and PC_{exp} *versus* $\bar{k}^{0.25}/C^{0.5}$ for the GHRF-related peptides, chromatographed under conditions of varied t_G and constant F, are shown in Fig. 7a and b, respectively. In both cases, it can be seen that PC increases linearly over the range $2 < \bar{k}^{0.25}/C^{0.5} < 4$ and that the PC values for peptide GHRF-3 are slightly lower than those for peptides GHRF-1 and -2. However, at longer gradient times, the magnitude of the experimental peak capacity was significantly lower than the calculated PC value.

The influence of flow-rate on the peak capacity, PC, for the LHRH- and GHRF-related peptides is shown in Figs. 8 and 9. Again, the similarity in the behaviour of the peptides LHRH-2, -4 and -6 is evident: PC increased to a maximum

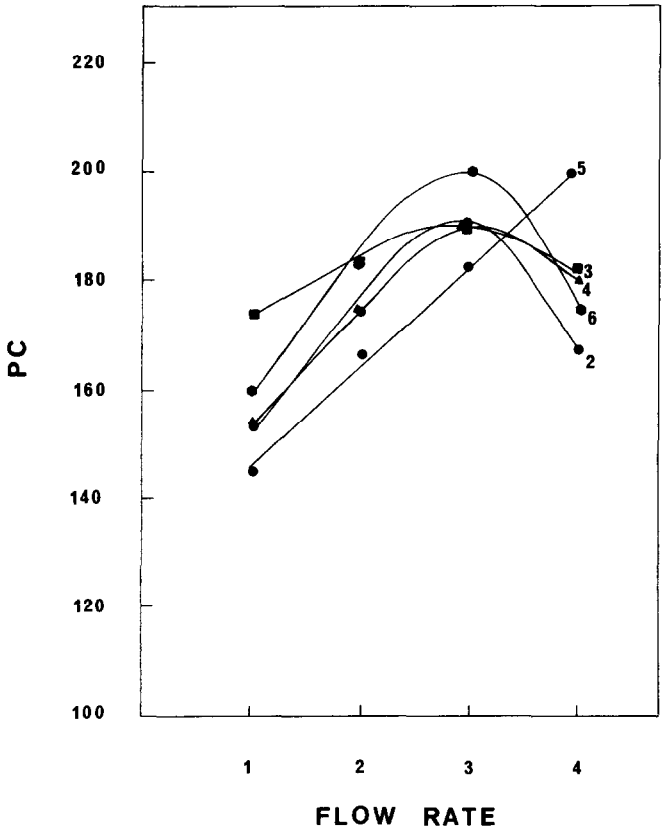

Fig. 8. Variation of peak capacity (PC) with flow-rate (F, in ml/min) for the LHRH-related-peptides LHRF-2–LHRH-6. The data correspond to calculated values obtained for the varied flow-rate experiments, where t_G = 40 min.

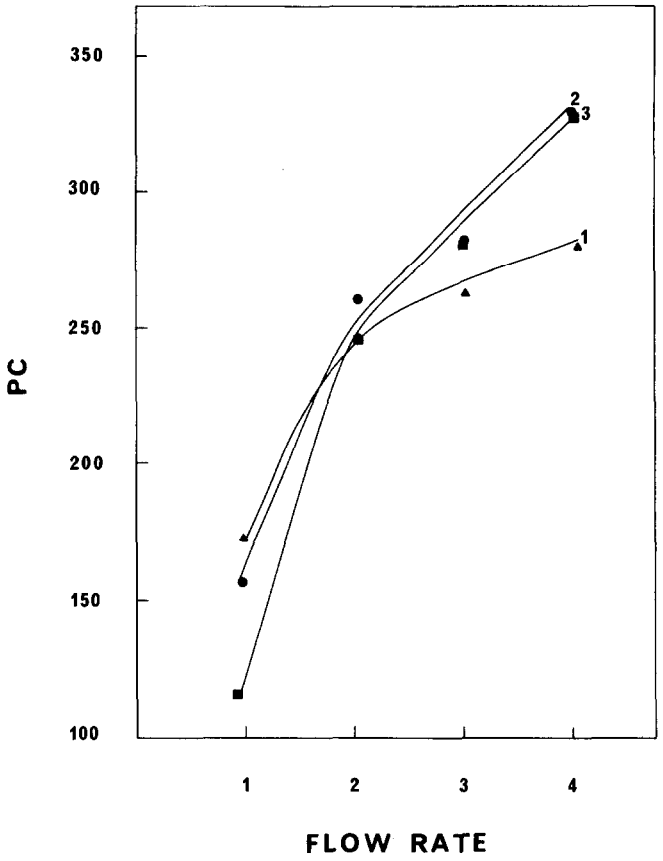

Fig. 9. Variation of peak capacity (PC) with flow-rate (F, in ml/min) for the GHRF-related peptides GHRF-1–GHRF-3. The experimental conditions are described in the legend for Fig. 8.

at $F = 3$ ml/min and then decreased at higher flow-rates. In contrast, the PC for peptide LHRH-5 increased up to the highest flow-rate employed in this study. The observed PC *versus* F behaviour of the peptides GHRF-1 -2 and -3 was essentially the same, tending to approach a maximum at the highest flow-rate examined. The inescapable conclusion from these results with these two sets of peptides is that at both low and high flow-rates under conditions of changing $\bar{k}$ (or b value), peak capacity for a specific peptide in a mixture may be far from optimal. Meek and Rossetti[27] have also noted similar peak capacity dependencies on flow-rate for insulin. Under the most optimal separation conditions, similar PC *versus* F and PC *versus* $1/b$ plots may be obtainable. In all other cases, it may not be feasible to obtain maximum PC values for each component of a mixture. The above results suggest that the nature of the band-broadening process within the column cannot be solely accounted for by kinetic models which assume that the solute behaves as a single conformationally rigid species throughout its entire passage along the chromatographic column. The observed differences in the PC plots as the experimental conditions are varied can thus be considered to accurately reflect the molecular similar-

ities or dissimilarities of the retention process itself for a series of peptide analogues as they interact with a hydrophobic surface. When the molecular mechanisms of interaction for a set of peptides are equivalent, the form of the PC plots for different members of the peptide set should bear very close similarities. When viewed in this way, the shape of the PC *versus* $k^{0.25}/C^{0.5}$ or PC *versus* F plots for the different LHRH- and GHRF-related peptides (and for other peptides in general) can thus be used to provide not only useful insight into the quality of the separation *per se*, but also into the question whether the retention and kinetic behaviour originates in the same or different mechanistic pathways of interaction which involve the solute and the stationary phase.

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