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

XL*. FURTHER STUDIES ON THE ROLE OF THE ORGANIC MODIFIER IN THE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF POLYPEPTIDES. IMPLICATIONS FOR GRADIENT OPTIMISATION

MILTON T. W. HEARN* and BORIS GREGO
St. Vincent's School of Medical Research, Victoria Parade, Fitzroy, Victoria 3065 (Australia)

SUMMARY

The retention behaviour of a variety of polypeptide hormones on octadecylsilica columns has been investigated as the volume fraction, φ_s, of the organic solvent modifier, acetonitrile, was systematically varied over the range $0 < \varphi_s < 0.8$ under low pH, low ionic strength conditions. The results are compared with data obtained in related experiments using eluents of the same pH containing 15 m*M* triethylamine. Under both sets of conditions non-linear dependencies of the logarithmic capacity factor, log k', values for these polypeptides, as well as for several phenylalanine oligomers, on φ_s were observed. In all cases examined the plots of log k' versus φ_s were bimodal with selectivity reversals from a dominant reversed-phase elution mode to a polar phase elution mode occurring at φ_s values near to 0.5 for the acetonitrile-based eluents. Within the range of k' values of interest in isocratic or gradient optimisation of reversed-phase separations of polypeptides, namely $1 < k' < 10$ with water-rich eluents of low pH, the dependency of log k' on φ_s has been evaluated in terms of a linear relationship approximation. Compared to small polar molecules, the slope, the values of the solvent strength parameters (s), and the extrapolated k' value intercepts at $\varphi_s = 0$, namely the k'_w values, derived from this linear relationship approximation, are considerably larger with peptides and polypeptides. Furthermore, the magnitudes of the extrapolated k'_w values and the estimated s values over this restricted range of φ_s values essentially follow the relative hydrophobicities of the polypeptides examined. The consequences of this retention behaviour on the choice of optimal gradient elution conditions for the separation of polypeptide mixtures on alkylsilicas are examined. In particular, the relationship between s value, gradient steepness and flow-rate, and the influence these parameters have on resolution and peak capacity under linear solvent strength conditions, have been further evaluated.

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INTRODUCTION

Despite the now widespread application of reversed-phase high-performance liquid chromatography (RP-HPLC) methods for peptide and polypeptide analysis, micropreparative separation and recovery (for a recent review, see ref. 1), experience on the optimisation of the stationary and the mobile phase parameters to suit a particular set of peptide, polypeptide or protein characteristics is still limited. Irrespective of the mobile phase pH or ionic strength, peptides, polypeptides and proteins are generally well retained on alkylsilicas when neat aqueous eluents are used. Further, with complex mixtures of these substances, isocratic elution conditions with aquo-organic solvent combinations rarely allow adequate resolution or recovery of all the components. Under gradient conditions small peptides differing widely in hydrophobicities and some polar polypeptides and proteins up to *ca.* 250 residues can, however, be successfully resolved with good recoveries on 5- or 10-μm chemically bonded alkylsilicas with nominal pore diameters typically in the range 6–10 nm. Less polar polypeptides and proteins often exhibit fickle chromatographic behaviour, such as low recoveries, poor peak shape or ghost peaks, when subjected to similar chromatographic conditions. The reasons for this behaviour are poorly understood although it is now well recognised that the retention of polypeptides and proteins to alkylsilicas is generally very responsive to small changes in the water content of the mobile phase. In earlier studies[2–5] we demonstrated that changes in peptide and polypeptide retention seen with reversed-phase systems as the mobile phase water content, ionic strength or pH are changed, or on the addition of hydrophilic or hydrophobic ionic additives to the eluent, could to a large extent be rationalised in terms of a dual, or two-site, adsorption model. The basis of the retention mechanism was believed to arise from composite hydrophobic (solvophobic) phenomena and polar (silanophilic, hydrogen bonding) phenomena established between the solutes and the stationary phase. With the water-rich eluents, the hydrophobic component dominates the retention process with the consequence that under these conditions, the retention order of a series of peptides can to a large extent be predicted on the basis of the summated effect of appropriate hydrophobic topological indices[6–8]. Furthermore, over limited ranges of mobile phase compositions, *e.g.* over a 20% incremental range in the organic modifier concentration, essentially linear dependencies of the logarithmic capacity factor on the surface tension of the mobile phase have been observed[2]. However, with water-lean eluents and some alkylsilica stationary phases, it is possible to obtain elution orders for unprotected peptides and polypeptides characteristic of a normal or polar phase elution mode. The purpose of the present study was to examine further the influence on polypeptide selectivity with a commercially available octadecylsilica reversed-phase packing as the volume fraction of the organic modifier, φ_s, is changed under more closely controlled ionic strength and pH conditions. In addition, the approach advanced by Snyder[9] for gradient optimisation for small molecules under linear solvent strength conditions has been adapted to the reversed-phase separation of polypeptides for mobile phases encompassing the range *ca.* $0 < \varphi_s < 0.5$. The results confirm that polypeptide resolution under these conditions is dependent on the gradient steepness with peak capacity varying in a predictable asymptotic manner.

EXPERIMENTAL

Chemicals and reagents

Water was quartz-distilled and deionised using a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Acetonitrile was HPLC grade obtained from Waters Assoc. (Millford, MA, U.S.A.) or Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Orthophosphoric acid, sulphuric acid, sodium sulphate and triethylamine were all AnalaR grade from BDH (Poole, Great Britain) or May and Baker (Dagenham, Great Britain). The sources of the polypeptides used in this study have been given previously[2,3]; several were subsequently repurified by reversed-phase procedures in this laboratory. All amino acids except glycine had the L-configuration.

Apparatus

All chromatographic data were collected with one (isocratic) or two (gradient) Model M6000A solvent delivery pumps, a M660 solvent programmer, a U6K universal chromatographic injector, a Model M450 variable-wavelength UV monitor and a M720 data module, all from Waters Assoc., coupled to an Omniscribe dual channel recorder. Sample injections were made with SGE Model 50A syringes (Melbourne, Australia). The pH measurements were performed with a Radiometer PHM-64 meter equipped with a combination glass electrode. The μBondapak C_{18} columns were purchased prepacked from Waters Assoc.

Methods

Bulk solvents and appropriate mobile phases were prepared and degassed by sonication as reported previously[2]. The μBondapak C_{18} columns were equilibrated with new mobile phase conditions for at least 30 min (*ca.* 50–100 column volumes). All chromatograms were carried out at 18°C. Sample sizes varied between 2 and 5 μg of peptide material injected in volumes ranging between 2 and 5 μl. The capacity factors for isocratic experiments and apparent capacity factors for gradient elution experiments were calculated by established methods using $NaNO_3$ to calibrate the column. All data points represent the average of triplicate measurements. A peak asymmetry algorithm[10] was used for the calculation of the peak standard deviation (in time units). The precision of the measurements was generally $\pm 2\%$. The s values for the various polypeptides were calculated from linear regression analysis of the isocratic retention data using a Hewlett-Packard 97 calculator. No significant differences in the s values were observed when capacity factors (k') calculated from peak maxima or first moments were used.

RESULTS AND DISCUSSION

Theoretical considerations

The variation of solute retention in RP-HPLC with solute structure and mobile-phase composition has been extensively reviewed[9,11,12]. Provided the ionisation state, extent of solvation and buffer ion interactions remain essentially constant over the range of solvent compositions examined, then essentially linear dependencies of $\log k'$ on φ_s have been observed for a variety of solutes over the range of k' values of interest in isocratic or gradient optimisation, *e.g.*, $1 < k' < 10$, although the de-

pendency of log k' on φ_s in isocratic RP-HPLC is usually considered to be quadratic[11,13]. Under such retention limits, the relationship between k' and φ_s for isocratic elution can be expressed in the familiar form discussed initially by Snyder and coworkers[9,14] for neutral and anionic solutes, namely:

$$\log k'_i = \log k'_{i,w} - s_i\varphi \tag{1}$$

where $k'_{i,w}$ is the capacity factor of the solute, P_i, at $\varphi = 0$, *i.e.*, in pure water, and s_i is the solvent strength parameter for P_i which will depend on the molecular characteristics of the organic modifier employed as well as the solute molecules.

Similarly, for linear solvent strength (LSS) gradient elution where log k' varies linearly with time, the capacity factor at the column exit, $k'_{i,e}$, can be given by[9]:

$$\log k'_{i,e} = \log k'_{i,w} - b\left(\frac{t_g - t_0}{t_0}\right) \tag{2}$$

where t_0 is the retention time of an unretained solute, t_g is the retention time of the retained solute, P_i, and b is the gradient steepness parameter. By definition, b is constant for all compounds eluted with a particular LSS gradient. With the organic solvents commonly used for polypeptide separation on alkylsilicas, namely methanol, acetonitrile, 1- and 2-propanol, the upper limit of organic modifier is usually limited in binary mobile phases by solubility considerations to less than *ca.* $\varphi_s = 0.6$, with a typical gradient condition for, say, a tryptic digest of a protein encompassing the range $0 < \varphi_s < 0.5$ (ref. 15). It can be seen from eqn. 2 that when k' values for peptides are small (*e.g.* less than 5), such peptides will be expected to chromatograph under gradient conditions essentially as an isocratic separation, and in fact isocratic conditions with hydrophobic pairing ion systems may be required to achieve adequate resolution. Such behaviour has been observed with small basic peptides, *e.g.* Ala-His-Arg (hGH-T-3), pGlu-His-Pro-NH_2 (TRF) or Ala-His-Gly-Lys (HbAβT-7), under low pH conditions.

Since resolution in gradient elution can be defined by:

$$R_s = 1/4\ (\alpha_g - 1)\ (N)^{1/2}\ Q \tag{3}$$

where $Q = \bar{k}'_i/(1 + \bar{k}'_i)$ (4)

$= 1/(2.3b + 1)\ G$ (5)

and α_g is the gradient separation factor ($k_{i,w}/k_{j,w}$) formally equivalent to the separation factor in isocratic elution, $\bar{k}_i$ is the median capacity factor value defined as the instantaneous capacity factor of the solute P_i as it reaches the midpoint of the column, and G is the band compression factor[9], itself a function of b. In gradient elution, the factor G arises as a consequence of the increase in solvent strength across the solute zone as it migrates through the column. The bandwidth of the eluting zone will thus depend on the b value of the system, becoming narrower at larger b values, although under usual gradient conditions the factor G will be close to unity. Resolution is thus anticipated to vary as an asymptotic dependency on b. For a given gradient separation with the b values for each component solute equal, the peak widths σ of the various elution zones will also remain roughly constant. The peak capacity (PC) for a gradient chromatographic separation whereby $R_s = 1$ for all adjacent zone pairs can be given by:

$$\mathrm{PC} = \frac{t_g - t_0}{4\sigma_t} \tag{6}$$

where t_0 and t_g are the gradient elution times for the first and last eluting peaks. As Snyder has shown[9] peak capacity can be expressed in the form:

$$\mathrm{PC} = c \cdot \Delta S \cdot Q \tag{7}$$

where c is a proportionality constant, Q is defined by eqn. 4 and ΔS is the difference in solvent strengths between elution condition A and elution condition B. Thus, peak capacity can be expressed as a function of the gradient steepness parameter by:

$$\mathrm{PC} = \frac{c \cdot \Delta S}{(2.3b + 1) \cdot G} \tag{8}$$

Thus, for a given set of separation conditions, peak capacity is anticipated to show an asymptotic behaviour as b is varied. Up to *ca.* $b = 1.5$, this behaviour will be progressively manifested as a gradual decrease in peak capacity (and hence loss in resolving power of the system) with significant decreases in peak capacity evident above *ca.* $b = 2.0$.

Detection sensitivity and peak height are inversely proportional to bandwidth. It is well recognised that the relative sensitivity of a band decreases with increasing k' in both isocratic and gradient elution. For gradient conditions, this can be defined in terms of a sensitivity factor, s_g, given by:

$$s_g = 2.3b\,Q \tag{10}$$

$$= \frac{1}{(1 + k'_e)\,G} \tag{11}$$

Hence, as b values increase with concomitant greater band compression, relative peak heights are also expected to increase. However, owing to a combination of thermodynamic and kinetic secondary phenomena, a practical limit will arise with very steep gradients as far as the potential benefits for detection sensitivity from band compression effects. The dependencies between detection sensitivity, recovery and the rate of change in mole fraction of the organic modifier with time, *i.e.*, $\mathrm{d}\varphi/\mathrm{d}t$, will be particularly noticeable with multi-component polypeptide mixtures in which the concentration of the individual components vary widely. For example, in trace enrichment of polypeptides from biological extracts, the limits of solubility of a particular component may be exceeded prior to its desorption from the stationary phase with steep inappropriate gradients and this will be reflected in poor recovery and detection sensitivity.

Since LSS gradients are usually achieved by continuously varying the mobile phase composition by changing φ_s in such a way that log k' changes linearly with time, *i.e.*, the gradient steepness parameter, b, remains constant, then the solvent strength parameter, s_i, for the polypeptide solute, P_i, and the gradient steepness parameter, b, can be interrelated through the equation:

$$b = s_i V_m \frac{\theta}{F} \tag{12}$$

where V_m is the mobile phase volume in the column (column dead volume), F is the volumetric flow-rate (and $V_m/F = t_0$) and θ is the rate of change of φ_s with time, *i.e.*, the gradient slope $d\varphi/dt$. Several corollaries arise from this relationship. Firstly, a decrease in gradient time by a factor of, say, two, whilst maintaining s_i, V_m and the limits of φ_s constant, will require a corresponding increase in the volumetric flow-rate in order to maintain a constant b-value. Secondly, if V_m is increased, *e.g.*, by using a column of longer length, then either θ must be decreased or F increased by the same factor if the b value is to remain constant. The converse would apply to columns of smaller dimensions. Thirdly, as s values become progressively larger, then for fixed F, either V_m or θ have to be progressively reduced to maintain b values relatively constant. The use of shorter alkylsilica columns would result in reduced V_m, but this will also result in reduced efficiencies (at fixed F) and lower sample capacity. For polypeptides or proteins with very large s values, these reductions may not present significant disadvantages for analytical separations, but clearly from a practical position it would be preferable in isolation studies to take advantage of shallow θ changes across the φ_s range of interest. Such shallow θ changes would be expected to permit improved resolution (at the expense of separation time), possibly improved recoveries owing to more favourable control over solubility parameter dependencies, but also reduced detection sensitivity.

Influence of the organic solvent modifier

Figs. 1–3 show data obtained with a variety of polypeptides (Table I) ranging in size up to 129 residues (hen lysozyme) as the volume fraction of acetonitrile is varied up to $\varphi_s = 0.8$. The primary eluents were either aqueous 4 m*M* sodium sulphate–15 m*M* orthophosphoric acid, pH 2.2, (elution system 1) or 4 m*M* sulphuric acid–15 m*M* orthophosphoric acid–15 m*M* triethylamine (elution system 2). These two conditions were chosen to permit an evaluation of the log k' versus φ_s dependency in presence and absence of a fixed concentration of triethylamine with both the pH and ionic strength essentially fixed. Adequate control over the pH is important in RP-HPLC separations. Differences in the pH of the eluent can lead to significant changes in the retention behaviour of most polypeptides, including selectivity reversals[1,7,16]. As is evident for all cases shown in Figs. 1–3, the plots of log k' *versus* φ_s for these acetonitrile systems are non-linear and pass through minima. The similarities of the plots obtained in the presence and absence of 15 m*M* triethylamine under these elution conditions exclude the possibility that the observed non-linear bimodal dependencies arise as a fortuitous consequence of subtle ionisation changes in the solutes with eluents of different solvent composition. Rather, they suggest that the phenomenon is due in part to changes in the relative populations of accessible "solvophobic" and "polar" binding sites on the stationary phase as the water-content is varied. With water-lean eluents, *i.e.*, $0.5 < \varphi_s < 0.8$ for the above acetonitrile conditions, few 'solvophobic' sites are apparently available with the consequence that polar-phase selectivities are observed. Depending on the nature of the silica matrix, *e.g.*, the presence of trace-metal contamination, more open siloxane network, changes in the shape of the log k' *versus* φ_s plots should thus arise. Data obtained with stationary

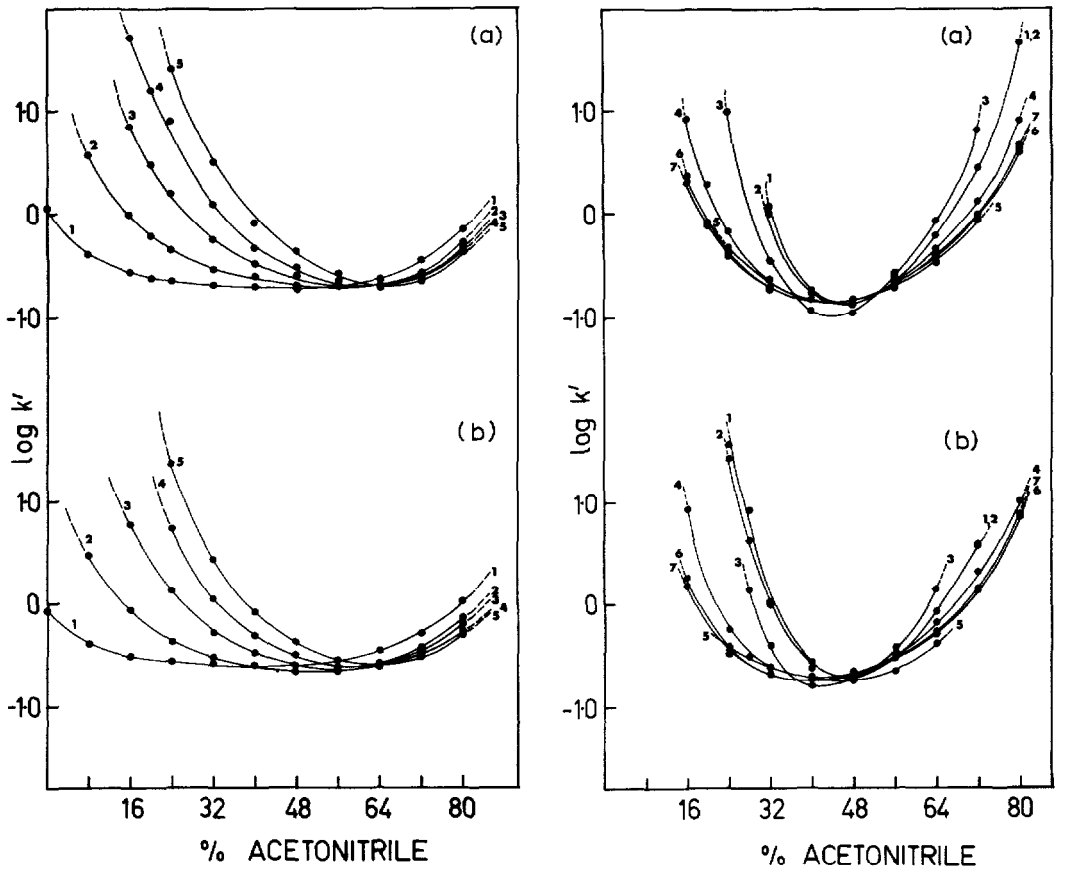

Fig. 1. Plots of the logarithmic capacity factors for several phenylalanine oligomers against the volume fraction, φ_s, of the organic solvent in water–acetonitrile isocratic mobile phases. Conditions: column, μBondapak C_{18}; flow-rate, 2.0 ml/min; primary mobile phases, (a) water–4 m*M* sodium sulphate–15 m*M* orthophosphoric acid, pH 2.2, and (b) water–4 m*M* sulphuric acid–15 m*M* orthophosphoric acid–15 m*M* triethylamine with the acetonitrile content adjusted over the φ_s range 0.0–0.8. The phenylalanine oligomer key is: 1 = F; 2 = FF; 3 = FFF; 4 = FFFF; 5 = FFFFF. See Table I for the one-letter amino acid code and relative hydrophobicities.

Fig. 2. Plots of the logarithmic capacity factors for several polypeptide hormones against the volume fraction, φ_s, of the organic solvent in water–acetonitrile isocratic mobile phases. The chromatographic conditions are the same as in the legend to Fig. 1 with the acetonitrile content adjusted over the φ_s range 0.16–0.80. The polypeptide key is: 1 = β-Leu^5-endorphin; 2 = β-Met^5-endorphin; 3 = porcine ACTH (1–39); 4 = angiotensin I; 5 = oxytocin; 6 = angiotensin II; 7 = angiotensin III.

phases of different matrix or ligand characteristics appear to be in agreement with this conclusion[17]. This suggests that it will be possible to design "specialty" non-polar stationary phases which amplify the reversed-phase or polar-phase aspect of the support thus enhancing selectivity for a particular component.

Although the above data clearly indicate non-linear dependencies of log k' on φ_s over the range $0 < \varphi_s < 0.8$, in the range of acetonitrile concentrations where regular reversed-phase retention behaviour is evident, that is over the φ_s range where

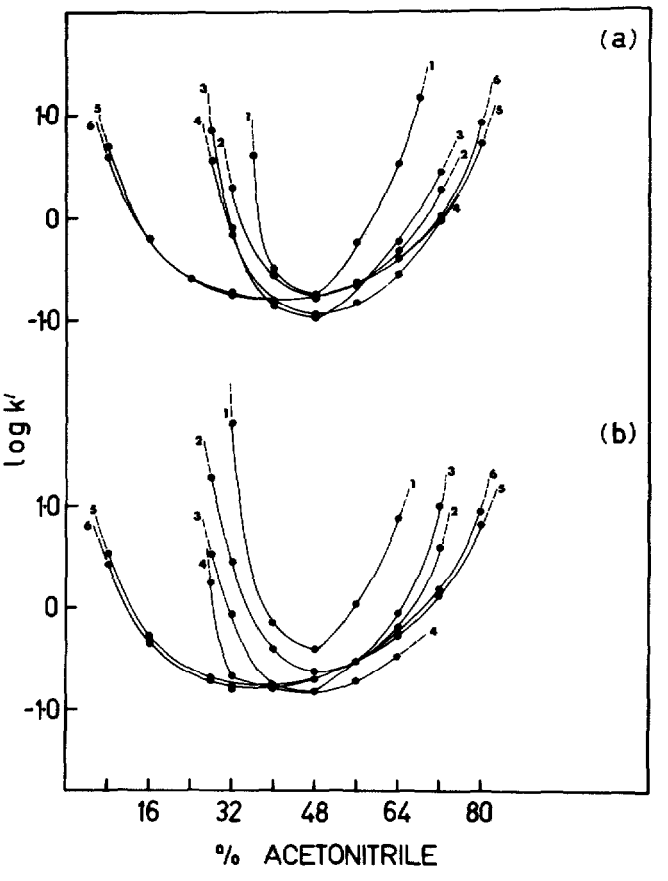

Fig. 3. Plots of the logarithmic capacity factors for hen lysozyme and several hormonal polypeptides against the volume fraction, φ_s, of the organic solvent in water–acetonitrile isocratic mobile phases. The chromatographic conditions are the same as in the legend to Fig. 1 with the acetonitrile content adjusted over the φ_s range 0.08–0.80. The polypeptide key is: 1 = hen lysozyme; 2 = porcine glucagon; 3 = bovine insulin; 4 = bovine insulin B-chain; 5 = arginine vasopressin; 6 = lysine vasopressin.

ca. $0.3 < k' < 5$, the relationship between log k' and φ_s can be approximated to a linear behaviour. It is apparent from analysis of this and other data[4,18] that the apparent slopes or s values increase rapidly as the relative hydrophobicities of the polypeptides increase and that s values for polypeptides are considerably larger, in several cases by a factor of 20 or more, than those seen for small polar organic molecules, or amino acids[14,18]. Besides implying very large k'_w values, large s values have other important implications. As has been observed with polypeptides and proteins, severe solvent dependencies, *i.e.*, large s values, will result in a very narrow operational range of solvent strength conditions with very little isocratic elution development possible. This elution behaviour can be considered an extension of a displacement mode. In practice, this effect can be used to advantage in batch extraction of polypeptides with bulk alkylsilicas thus allowing rapid fractionation of biological extracts. As expected, the smaller, more polar polypeptides exhibited the smallest incremental changes in their log k' values as the acetonitrile content was increased to $\varphi_s = 0.5$. In the context of low pH elution conditions, this behaviour will generally

TABLE I

PEPTIDES AND POLYPEPTIDES USED IN THE PRESENT STUDY*

Peptide	*Number of residues*	*Relative hydrophobicities*		*Calculated§ retention parameters*			
				Elution system 1		*Elution system 2*	
		Σf**	ΣX***	$\ln k'_{i,w}$	S_i	$\ln k'_{i,w}$	S_i
F	1	2.24	4.06	§§	–	§§	–
FF	2	4.48	6.58	0.9	5.8	0.8	5.1
FFF	3	6.72	9.10	2.2	8.3	2.0	7.7
FFFF	4	8.96	11.63	3.3	10.5	2.8	8.9
FFFFF	5	11.20	14.15	4.3	12.2	4.5	12.9
RVYIHPF (Angiotensin III)	7	7.07	7.40	1.9	10.0	1.9	11.0
DRVYIHPF (Angiotensin II)	8	7.05	9.32	2.2	11.2	2.0	11.0
CYIQNCPLG (Oxytocin)	9	6.77	5.16	n.d.	n.d.	n.d.	n.d.
CYFQNCPKG (Lysopressin)	9	5.55	−1.81	1.4	10.6	1.2	10.5
CYFQNCPRG (Argpressin)	9	3.93	−2.65	1.6	11.0	1.4	11.2
DRVYIHPFHL (Angiotensin I)	10	8.81	9.80	3.4	15.4	3.6	17.4
Porcine glucagon	29	8.49	14.38	5.1	15.0	5.6	15.8
Bovine insulin B-chain	30	15.75	12.89	5.7	18.5	4.7	16.0
β-Met^5-endorphin	31	15.90	15.93	3.4	10.7	5.7	17.3
β-Leu^5-endorphin	31	16.87	15.53	4.0	12.5	5.8	17.0
Porcine ACTH (1–39)	39	23.52	16.61	6.6	23.5	4.6	16.0
Bovine insulin	51	26.47	27.73	6.7	21.1	5.3	17.0
Hen lysozyme	129	42.41	27.96	10.4	27.5	10.7	28.0

* The one-letter code for the amino acids is as given by M. O. Dayhoff in *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Silver Spring, MD, 1972.

** Calculated using f(amino acid) values from ref. 25 with f(glycine) = 0 and f(arginine) = −1.10, respectively.

*** Calculated using X (amino acid) values from ref. 8.

§ Calculated according to eqn. 1 by linear regression analysis of the retention data obtained over the range *ca.* $1 < k' < 5$; regression coefficients were greater than 0.93.

§§ $\ln k' < 0.05$; n.d. = not determined.

be most evident for basic peptides, with the opposite effect applying with acidic peptides at pH values near pH 7.5.

The separation on alkylsilicas of biological mixtures of polypeptides encompassing even a modest range of hydrophobicities will almost certainly require gradient elution, and if high resolution and recovery with short analysis times are mandatory, then shallow gradients with relatively high flow-rates will be required. In accord with eqns. 10 and 12 higher flow-rates in the gradient elution of the polypeptides studied resulted in decreased detector sensitivity. Loss of resolution was not, however, as significant as seen in isocratic separations. Interestingly, high flow-rates under gradient conditions gave higher mass recoveries with several of the polypeptides, an effect also seen with proteins[19].

In gradient elution, the apparent capacity factor, $k'_{i,\mathrm{app}}$, for a polypeptide, P_i, which elutes from a reversed-phase column with a gradient time, $t_{g,i}$, can be defined[9] as:

$$k'_{i,\mathrm{app}} = (t_{g,i} - t_0)/t_0 \tag{13}$$

$$= \left(\frac{1}{b}\right) \log (2.3b\, k'_{i,w} + 1) \tag{14}$$

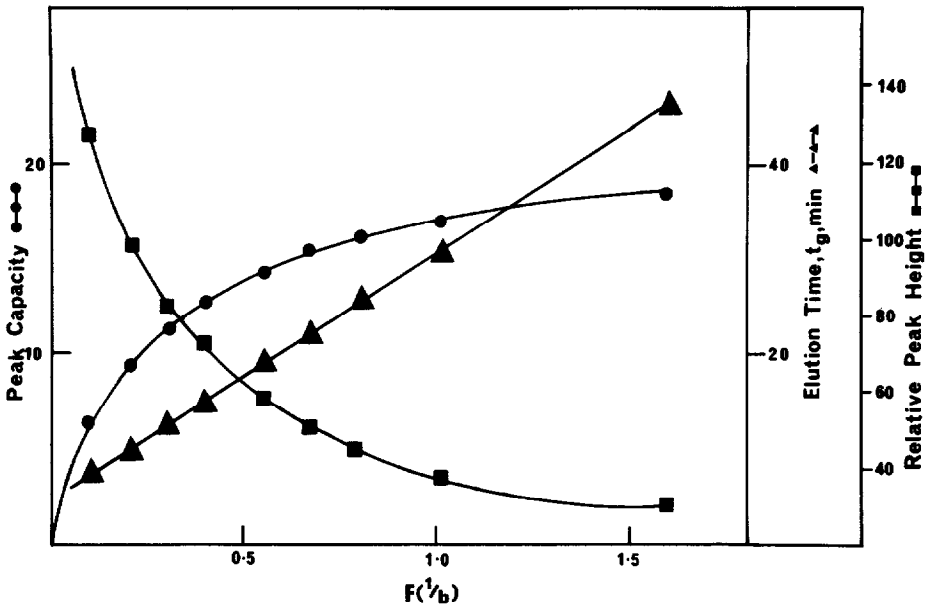

Fig. 4. Plots demonstrating the relationships for peak capacity, PC, elution time, t_g, and relative peak height as a function of the reciprocal of the gradient steepness parameter, b, for penta-L-phenylalanine. The gradient delay time has not been subtracted from the measurements of the various elution times, although this is automatically deleted for calculations of peak capacity. Conditions: column, μBondapak C_{18}; temperature, 18°C; eluent A, 25 m*M* potassium dihydrogen phosphate–15 m*M* orthophosphoric acid; eluent B, 50% acetonitrile–50% water–25 m*M* potassium dihydrogen phosphate–15 m*M* orthophosphoric acid; sample loading, 5 μg of penta-L-phenylalanine and phenylalanine in 5 μl. The gradient slope and/or flow-rate were adjusted as appropriate to each experiment.

For most of the polypeptides examined in this study, the extrapolated $k'_{i,w}$ values were large, *i.e.*, greater than 1000. As a consequence, the dependence of the apparent capacity factor on the gradient steepness parameters can be expressed by the reduced form of eqn. 14, namely:

$$k'_{i,app} = \left(\frac{1}{b}\right) \log k'_{i,w} \tag{15}$$

Thus for peptides, polypeptides and proteins where s values (and hence intrinsic k'_w values) are large, shorter relative gradient retention can be achieved by increasing the b values. However, as k'_{app} values are reduced so also will be the $\bar{k}'$ values, and resolution will suffer particularly for those earlier eluting components with smaller intrinsic $\bar{k}'$ values. In order to achieve gradient optimisation for a complex mixture of polypeptides, chromatographic conditions need to be chosen whereby control over the b values, the s values, the volumetric flow-rate, F, the phase ratio in the column (and specifically the mobile phase volume, V_m), and the gradient slope $d\varphi/dt$ allows all the components in the mixture to elute over the range of *ca.* $0.3 < \log k'_{app} < 1.3$. When the resolution of polypeptides within a particular range of molecular hydrophobicities alone is considered, the choice of the initial solvent composition is thus of major importance.

As discussed above, resolution in gradient elution separations of polypeptides should improve as the gradient steepness decreases, but concomitantly relative peak heights will become reduced (eqns. 3 and 10). The effect of gradient steepness and flow-rate on resolution (as assessed from peak capacity) and relative peak height was examined and representative experimental data for the hydrophobic pentapeptide, $(Phe)_5$, is shown in Fig. 4. In accord with eqn. 15, a linear inverse relationship between gradient elution time and gradient steepness was observed for this polypeptide although in several other cases, *e.g.*, glucagon and lysozyme, shallow inverse curvilinear dependencies were noted. The anticipated behaviour of resolution increasing, and peak heights decreasing, as the gradient steepness parameter, b, becomes smaller is clearly evident. These results are in accord with the recent observations by Meek and Rossetti[6] for peptides separated on a Bio-Rad ODS column with a sodium perchlorate–orthophosphoric acid–acetonitrile gradient system, and of Cohen and Karger[20] for several proteins separated with a 1-propanol gradient system. Furthermore, when flow-rate and gradient slope, θ, were varied in tandem so that the b value remained constant, then the respective peak heights for the various polypeptides examined also remained essentially constant. With the smaller peptides used in the present study, flow-rate changes at constant b had only a relatively small effect on peak capacity, *e.g.*, with $(Phe)_5$ the PC increased from 10.5 to 11.2 when the flow-rate was halved. In agreement with our earlier observations[3], and the results of others[20,21], with larger polypeptides a lower flow-rate, *e.g.*, $F < 0.5$ ml/min, at constant b improved resolution but resulted in unacceptably long analysis times. Improved resolution was also evident with the larger polypeptides as θ values were reduced at a fixed flow-rate. This suggests that shallow gradients may, in general, be more appropriate for polypeptides with large k'_w and s values, particularly when suitable solvent strength limits can be chosen to allow reasonable separation times and recoveries. These conclusions are in basic agreement with the predictions of Dolan *et al.*[14] as well as other recent observations on the separation of hydrophobic polypeptides including haemoglobin fragments, apolipoproteins and thyroglobulin peptides[22–24].

In summary, the above experiments indicate that resolution of polypeptides on alkylsilicas under gradient conditions can be controlled through variation of the s values of the solutes as well as by variation of the gradient programme and flow-rate. The present study also confirms the utility of the linear solvent strength approach for the selection of optimal conditions for a particular gradient separation. For polypeptides (and proteins) with larger s values, very little elution development is expected under RP-HPLC conditions. Advantage of these effects can be taken with microparticulate, chemically bonded n-alkylsilicas and hybrid stationary phases with adequate surface properties to facilitate rapid sorption kinetics and high selectivity in the fractionation of polypeptides and proteins at the semipreparative, and larger, scale[24]. With sufficient data available on polypeptide s values and recoveries in different chromatographic systems, mobile phase selection for optimal conditions would be considerably simplified. One consequence of this data base would be a more rigorous optimisation scheme replacing the largely empirical trial-and-error approaches used currently in most RP-HPLC studies on the isolation of polypeptides from biological sources.

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