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

LXV*. STUDIES ON THE OPTIMISATION OF THE REVERSED-PHASE GRADIENT ELUTION OF POLYPEPTIDES: EVALUATION OF RETENTION RELATIONSHIPS WITH β-ENDORPHIN-RELATED POLYPEPTIDES

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SUMMARY

The gradient retention behaviour of 29 polypeptides related to β-endorphin has been investigated using a 15-nm pore diameter octadecylsilica stationary phase and 0.1% trifluoroacetic acid in water–acetonitrile as mobile phases. In particular, this study has examined the influence of changes in gradient time and flow-rate on relative retention, resolution, and bandwidth. The utility of quantitative expressions derived from linear solvent strength gradient theory has been further assessed. Evaluation of capacity factor (k') dependencies on the mole fraction of organic solvent (φ) has been used to determine the various solute parameters, including the S and $\log k'_0$ values from gradient data. Methods to adjust band-spacing via changes in gradient steepness parameters are documented. Peak capacities for various polypeptides have also been determined under different gradient conditions. The results demonstrate that solute-specific variation in peak capacity can arise, presumably due to anomalous band-broadening changes, even with small peptides under low pH gradient elution conditions with *n*-alkylsilicas. The determination of solute selectivity parameters (τ) as an adjunct to the characterisation of structural group effects with peptide analogues is examined. This information appears particularly useful for the evaluation in physicochemical terms of solute-solvent and solute-stationary phase interactions as well as other distribution processes associated with polypeptide retention to *n*-alkylsilicas.

INTRODUCTION

During the past decade there has been enormous growth in our knowledge of the structure and function of biologically active macromolecules. These advances may largely be attributed to the development of innovated techniques in electrophoresis and high-performance liquid chromatography (HPLC). Certainly, over this

* For Part LXIV, see ref. 1.

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period, reversed-phase (RP)-HPLC has come to assume a pivotal role in these scientific advances as the dominant technique for the purification of peptides and polypeptides from natural and synthetic sources. Various reasons can be advanced for the popularity of RP-HPLC techniques in polypeptide isolation and analysis, including (1) the excellent resolution which can be achieved for closely related, as well as structurally disparate, substances under a large variety of chromatographic conditions; (2) the experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase composition simply by the addition of different ionic and non-ionic additives; (3) the generally high recoveries, even at ultramicroanalytical levels; (4) the potential for evaluating different physicochemical aspects of solute–eluent or solute–stationary phase interactions from chromatographic data; and (5) the excellent reproducibility of repetitive separations, carried out over long periods of time, a feature reflecting the stability of the hydrocarbonaceous stationary phase to most mobile phase conditions below pH 7.5. These different capabilities of RP-HPLC have been amply demonstrated in numerous application studies on the analysis and purification of polypeptides (for compendia of applications see refs. 1–7).

Despite the now wide usage of RP-HPLC techniques for polypeptide separation, the selection of a particular chemically bonded hydrocarbonaceous stationary phase and mobile phase combination has frequently been based on empirical criteria. Not uncommonly, such arbitrary selections do not adequately address the issues of optimal chromatographic resolution, band shape, or solute recovery. Clearly, more information on stationary phase surface topography and the physicochemical basis of polypeptide retention in reversed-phase systems is required before these empirical approaches can be replaced by a more systematic strategy. In recent investigations (see, *e.g.*, refs. 8–15) from this and other laboratories the influence of mobile phase composition on the chromatographic behaviour of polypeptides separated on meso- and macro-porous, chemically bonded *n*-alkylsilicas has been documented. Other studies (*e.g.* refs. 16–21) have examined the effect of stationary phase characteristics, including the particle size, the mean pore diameter, the porosity, the accessible surface area, the ligand composition and surface density, and the silica treatment history on polypeptide resolution and recovery in RP-HPLC systems. Column configuration[13,22,23], mobile phase flow-rate[8,20], temperature[23,24] and sample size[17,23] are also known to affect both of these crucial aspects of a successful purification.

Collectively, these investigations have revealed that the separation of polypeptides on *n*-alkylsilicas can involve chromatographic distribution and kinetic phenomena which are much more complex than those shown by small, polar molecules. These expressions of polypeptide chromatographic behaviour in reversed-phase systems are believed to be due to composite effects, arising from specific solute solvation equilibria[25], from multisite interactions with the heterogeneous stationary phase surface[11,12], from solute aggregation in the bulk mobile phase or at the stationary phase surface[23,24], from sol-gel equilibria[26], from specific ion-interaction equilibria involving ionic additives present in the mobile phase or adsorbed on the non-polar stationary phase surface[27] and from specific pH-dependent ionisation equilibria[27] which reflect the unique isoelectric point characteristics of these zwitterionic polyelectrolytes in solution. Despite the considerable differences which exist between the RP-HPLC behaviour of polypeptides and small polar organic molecules, recent experience in-

dicates that both isocratic elution theory and gradient elution theory, as developed for simple organic acids or bases, can be used to evaluate the retention behaviour of polypeptides, separated under regular reversed-phase conditions, provided the migration of each polypeptide solute along the chromatographic column can be represented by a single, conformationally stable species or, alternatively, by a time-averaged structure of rapidly interconverting conformers. Further adaptation of these theoretical treatments is required[28,29] in circumstances where the interconversion between different conformers of the same polypeptide occurs on a similar time scale to the overall separation time.

Although isocratic conditions can in many instances be employed in the separation of different polypeptides under well-defined reversed-phase conditions, complex mixtures of polypeptides are typically separated on *n*-alkylsilicas under gradient elution conditions. Starting with the elegant linear solvent strength gradient theory of Snyder[30], the present paper examines quantitative relationships for the relative retention and resolution of polypeptides, separated on *n*-alkylsilicas, using as test substances a series of β-endorphin-related polypeptides. Besides providing a detailed validation of this gradient model, the present investigation has also permitted comparison of functional group parameters and retention behaviour in gradient systems. The results demonstrate that changes in the gradient steepness parameter can be used rationally to adjust bandspacing. Further, the experimental data suggest that stationary phase-mediated dynamic effects may significantly influence band broadening, even with relatively small polypeptides, under isocratic and gradient elution conditions.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile was HPLC grade, obtained from Millipore (Lane Cove, Australia). Trifluoroacetic acid (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.). Various β-endorphin related peptides were either generously provided by Dr. J. van Nispen, (Organon, Oss, The Netherlands) or obtained from Peninsula Labs. (Belmont, CA, U.S.A.) and purified in this laboratory by reversed-phase chromatography. 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 U6K universal chromatographic injector, a Model M450 variable-wavelength UV monitor operating at 210 nm, all from Waters Assoc. Division, Millipore (Milford, MA, U.S.A.), coupled to a Hewlett-Packard 3390A integrator. Gradients were generated using a precalibrated Waters Assoc. M660 solvent programmer. 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.

Chromatographic procedures

Bulk solvents and mobile phases were degassed by sonication. Chromato-

graphic measurements were made at 20°C using 25 cm × 4.6 mm I.D. stainless-steel columns, packed with dimethyloctadecylsilica of mean particle diameter of 6 μm, specific surface area of 143 m^2/g with corresponding average nominal pore diameter of 13 nm. Based on carbon analysis, the ligand density of the stationary phase was 2.03 μmoles dimethyloctadecylsilyl groups per square meter. The columns were stored in methanol when not in use and equilibrated to new mobile phase conditions for *ca.* 60 min at a flow-rate of 2 ml/min. Sample sizes for each polypeptide in mixtures varied between 1 μg to 10 μg with injection volumes between 10–100 μl. The column dead-time, t_0, was taken as the retention time for sodium nitrate. All data points represented the average of at least triplicate measurements. The various chromatographic parameters (b, S, $\bar{k}'_0$, log k'_0, $\bar{\varphi}$, PC, R_s, t_g (calc), θ', β, τ) were calculated using the HAN-ChromoCalc programme, developed in this laboratory and written in BASIC language for Hewlett-Packard HP86B computer, using inpout values of t_{SEC}, t_0, F, t_g, V_m, V_0, t_G and $\Delta\varphi$.

RESULTS AND DISCUSSION

Retention relationships in gradient elution

Polypeptides frequently show very pronounced retention dependencies on the volume fraction, φ, of the organic solvent modifier under reversed-phase elution conditions. Not uncommonly, a very small change in φ (*e.g.*, less than 0.1) from some intermediate φ value will encompass the operational chromatographic retention range of $1 < k' < 100$ for a particular polypeptide. This dependency between the capacity factor, k', for a polypeptide, separated under regular reversed-phase isocratic elution conditions, and the volume fraction, φ, (or alternatively the solvent concentration, [solvent],) can be represented in specified chromatographic circumstances by the relationship

$$\log k'_i = \log k'_{0,i} - S\varphi \tag{1}$$

or

$$\log k'_i = C - m \log [\text{solvent}] \tag{2}$$

Over relative narrow φ value ranges, linear dependencies between log k' and φ are thus anticipated with intercept at $\varphi = 0$ or log k'_0 and slope S. Similarly, linear dependencies between log k' and [solvent] are expected with intercept at [solvent] $= 0$ of C and slope m. Experimentally, the plots of log k' *versus* φ (or [solvent]) have been observed[4,8,25,31] to be curved rather than linear. In these cases, the slope S simply represents the tangent to the curve at a specific k' or φ value. The S and m values of polypeptides are usually large, when compared to those of simple organic acids. Typically, S values greater than 20 have been observed for larger polypeptides and small proteins. Similarly, the calculated log k'_0 values for polypeptides are also much larger than obtained for small solutes. These features of polypeptide retention behaviour with *n*-alkylsilicas are believed to be consequences of multisite ligand–solute interactions[11,12] and reflect differences in the magnitude of the hydrophobic contact area, established between the solute and the hydrocarbonaceous ligand. De-

pending on the topography of the stationary phase surface, bimodal dependencies between log k' and φ can be obtained for polypeptides separated on *n*-alkylsilicas with selectivity reversals from a dominant reversed-phase elution order to a polar phase elution mode, occurring at characteristic φ values. In the general case with a defined column, eluent composition, temperature, and pH, and in the absence of slow interconversion between conformational species of a particular solute, the isocratic retention of small polypeptides on alkylsilicas over a wide range of organic solvent concentrations can be approximated by the empirical relationship

$$k' = \rho_s k'_{SEC} + \rho_r k'_0 e^{-S\varphi} + \rho_P k'_P e^{-D(1-\varphi)} \quad (3)$$

SEC term solvophobic term silanophilic term

where k'_{SEC}, k'_0 and k'_P are the capacity factors of the peptidic solute, P_i, due to size-exclusion (SEC) processes, or retention in neat water (or the initial mobile phase condition) and at the final organic solvent condition respectively; ρ_s, ρ_r and ρ_P are the weighted mole fractions of the solute associated with each retention mode, and the variables S and D are the tangents to the curves for the descending (solvophobic) and ascending (silanophilic) aspects of the log k' *versus* φ plot at specified k' and φ values. The S and D terms are both solute- and condition-dependent variables. As their magnitudes increase, the elution φ-window over which realistic k' values can be achieved will progressively narrow. With polypeptides which exhibit very large S and D values with a particular non-polar stationary phase, very precise selection of isocratic mobile phase compositions is required. Because of the strong dependence of retention time on mobile phase composition, very small changes in solvent content, pH, buffer concentration or temperature are reflected in large changes in k'. Frequently, these retention changes are also accompanied with significant increase in plate height and peak asymmetry thus leading to major degeneration of the chromatographic performance. In gradient elution, selection of the mobile phase composition is usually less demanding since a specific polypeptide tends to elute at the same organic modifier percentage irrespective of the choice of the initial and terminal mobile phase compositions provided these solvent conditions encompass the correct range of elution strengths. However, even in gradient elution the chromatographic behaviour of polypeptides can still be profoundly affected by secondary solution phenomena such as pH or buffer ion effects.

Evaluation of the solute variables S, D, k'_{SEC}, k'_0, k'_P, etc. is important for several reasons. Firstly, this information can be directly applied to the enhancement of resolution via optimisation procedures with a particular chromatographic system. Secondly, knowledge of these parameters greatly simplifies the determination of the physicochemical relationships underlying selectivity-functional group dependencies. At this stage, such use of RP-HPLC data with hormonal polypeptides has not been extensively investigated, although the potential for the design and purification of super potent analogues is now appreciated. Thirdly, analysis of these chromatographic variables provides quantitative guidelines required for the preparation of improved reversed phases, *i.e.* for the characterisation of different stationary phase topographies and effect of different column configurations. The task of determining the variables S, k'_{SEC}, and log k'_0 for a large variety of polypeptides encompassing differences in composition, sequence, size and hydrophobicity clearly requires the choice of dif-

ferent isocratic conditions such that polypeptide retention is dominated by solvophobic phenomena. Under such conditions, values of S, k'_{SEC} and log k'_0 can be derived for different chromatographic systems although reliable measurements of the necessary isocratic chromatographic data tend to be time-consuming and require high experimental precision. Furthermore, many polypeptides, when eluted under isocratic conditions from *n*-alkylsilicas, exhibit skewed peaks and, as a consequence, accurate determination of the average elution time and the peak variance requires computation of the first and second moments of the peak.

Because of these interrelated molecular and chromatographic complexities associated with the separation of polypeptides on *n*-alkylsilicas, recourse is usually had to gradient elution which provides a useful expedient to reduce separation times and decrease peak volume (equal to $4\sigma_v$). However, it is worth recalling that the same chromatographic variables which control retention, resolution, and bandwidth in isocratic elution are also relevant in gradient elution. If it is assumed that all prevailing secondary solution equilibria remain constant under gradient conditions of changing gradient time, t_G, flow-rate, F, or organic modifier mole fraction, φ, then, the retention time, t_g, for a polypeptide chromatographed under gradient elution conditions can be expressed according to the Snyder model[30] as

$$t_g = (t_0/b) [\log 2.3k_0b (t_{SEC}/t_0) + 1] + t_{SEC} + t_d \quad (4)$$

where t_0 is the column dead-time; k_0 is the capacity factor for the peptide solute in the initial conditions of the gradient; t_{SEC} is the retention time of the polypeptide solute solely under SEC elution conditions (or alternatively the retention time of a non-interactive solute of identical molecular mass and radius of gyration), t_d is the time required for the mobile phase to reach the column inlet as the volume fraction of the B solvent increases, and b is the gradient steepness parameter. By definition, linear solvent strength gradients require the value of b for all components to be constant. Usually, with most gradient systems as currently used, the value of the parameter b varies for different polypeptides. For a given column, *i.e.* a defined V_m and a fixed flow-rate, F, the parameter b is known from experimental and theoretical studies to be inversely proportional to the gradient time, t_G, and proportional to the change in the volume fraction of the B eluent per unit time, θ', ($= \Delta\varphi/t_G$). Consequently, the variables which quantitatively define the requirements of linear solvent strength gradients in reversed-phase separations can be interrelated through the expressions

$$b = SV_m \frac{\theta'}{F} = St_0 \frac{\Delta\varphi}{t_g} \quad (5)$$

It directly follows from eqn. 5 that the magnitude of the solute-dependent variable, S, (and hence from eqn. 1 the magnitude of the intercept term log k'_0) can be calculated using regression techniques from values of the parameter b, derived from experimental retention data. As demonstrated by Snyder and co-workers[13,30] the determination of the b values can be easily achieved by using gradients of different t_G and/or F. Thus for polypeptides with large k'_0 values (as is usually the case for most peptides separated on high-coverage *n*-alkylsilicas) the parameters $b_{1,i}$, $b_{1,j}$, $b_{1,k}$,

... $b_{1,n}$ for a specified gradient time, t_G, can be calculated from the gradient retention times $t_{g1,i}$, $t_{g1,j}$, $t_{g1,k}$ ... $t_{g1,n}$ of polypeptides P_i, P_j, P_k, ... P_n from two (or more) experiments of different gradient times t_{G1}, t_{G2}, t_{G3}, ... t_{Gn}, such that for polypeptide P_i

$$b_{1,i} = (t_{0,i} \log \beta)/[t_{g1,i} - (t_{g2,i}/\beta) + t_{0,i}(t_{G1} - t_{G2})/t_{G2}] \quad (6)$$

In circumstances when both gradient time and flow-rate are varied simultaneously, then the parameter b can be expressed in terms of the corresponding elution volumes for the different flow-rates F_1, F_2, F_3, ... F_n such that

$$b_{1,i} = -(\log \beta) \Big/ \left[\frac{(V_{g1,i} - V_{0,1})}{F_1 t_{0,1}} - \beta \frac{(V_{g2,i} - V_{0,2})}{F_2 t_{0,2}}\right] \quad (7)$$

where $\beta = t_{G2}/t_{G1}$, $V_{g1,i}$ and $V_{g2,i}$ are the gradient retention volumes for the polypeptide P_i, eluted at the flow-rates F_1 and F_2, and $V_{0,1}$ and $V_{0,2}$ are the retention volumes corresponding to a non-retarded component which is eluted in times $t_{0,1}$ and $t_{0,2}$ under the two different flow and gradient time conditions.

In gradient elution, the instantaneous capacity factor corresponding to the midpoint of the solute migration along the column can be represented by the medium capacity fractor, $\bar{k}'$, and the corresponding plate number and volume fraction of the organic solvent modifier at this point can be given by $\bar{N}$ and $\bar{\varphi}$, respectively. Variation of t_G (and hence change in b) in gradient elution will formally have the same effect on the $\bar{k}'$ *versus* $\bar{\varphi}$ relationships of a series of polypeptides, separated on *n*-alkylsilicas as an equivalent variation in φ will have on the k' values of the same solutes separated under isocratic elution conditions. Hence, in analogy to the way in which φ or solvent strength affects resolution in isocratic separations $\bar{\varphi}$ affects resolution in the gradient mode [assuming that selectivity ($\bar{\alpha}_g = \bar{k}'_i/\bar{k}'_j$) and $\bar{N}$ are maintained constant]. Since resolution in gradient elution can be defined as

$$R_s = \frac{1}{4}[(\bar{k}'_i/\bar{k}'_j) - 1] N^{1/2} [\bar{k}'_i/(1 + \bar{k}'_i)] \quad (8)$$

then for gradient systems of constant selectivity, optimisation of resolution for polypeptides P_i, P_j, etc., will be achieved when $\bar{N}^{1/2} - [\bar{k}'_i/(1 + \bar{k}'_i)]$ is maximised. It can be seen that in order to achieve optimal $\bar{N}$ values at a particular $\bar{k}'$ value it is essential that chromatographic conditions are selected whereby slow interconversion between different conformational states of a polypeptide and other unfavourable kinetic effects induced at the stationary phase surface are avoided. Previous studies[9,24,28,29,32] on band-broadening of polypeptides and proteins, separated on *n*-alkylsilicas, indicate that these dynamic effects can contribute significantly to decreases in resolution and may lead in the case of some polypeptides to multiple or composite skewed peaks for an apparently homogeneous substance.

It can be shown[30] that the median capacity factor in a linear solvent strength gradient system is given by

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

TABLE I

STRUCTURES AND RETENTION PARAMETERS OF β-ENDORPHIN-RELATED POLYPEPTIDES

Peptide	*Sequence*	*Position in β-endorphin*	*S**	*log k_0**	*MW*	χ_1**	χ_2**	χ_3**	χ_4**
1	YGGFM	1–5	10.00	7.26	573	5.02	5.19	34.9	26.2
2	YGGFMTS	1–7	11.19	7.67	761	4.20	5.06	31.6	21.3
3	YGGFMTSEKSQTPLVT	1–16	14.18	11.42	1744	6.94	7.13	45.0	28.1
4	YGGFMTSEKSQTPLVTL	1–17	13.13	12.60	1859	8.93	10.29	65.0	37.7
5	YGGFMTSEKSQTPLVTLFK	1–19	13.13	14.06	2134	11.69	12.28	80.5	47.3
6	YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE	1–31	15.08	18.62	3263	17.82	17.36	83.4	65.6
7	GGFM	2–5	8.96	5.71	410	3.32	5.98	29.0	19.5
8	GGFMT	2–6	9.70	5.80	511	3.06	5.19	29.8	17.8
9	GGFMTS	2–7	14.63	6.56	598	2.50	5.85	25.7	14.6
10	GGFMTSE	2–8	11.64	5.53	727	2.43	6.29	18.6	15.7
11	GGFMTSEK	2–9	10.90	5.80	855	2.95	5.76	14.9	12.7
12	GGFMTSEKSQ	2–11	13.28	7.03	1070	1.30	5.66	10.5	7.5
13	GGFMTSEKSQTP	2–13	14.33	8.32	1268	2.05	4.47	16.4	8.9
14	GGFMTSEKSQTPLVT	2–16	15.22	10.97	1581	5.24	7.92	40.7	20.1
15	GGFMTSEKSQTPLVTL	2–17	13.73	12.13	1694	7.23	11.08	60.7	30.7
16	GGFMTSEKSQTPLVTLF	2–18	12.84	13.63	1841	9.47	13.60	79.9	43.3
17	GGFMTSEKSQTPLVTLFK	2–19	14.03	14.17	1969	9.99	13.07	76.2	40.3
18	TSEK	6–9	***	***	463	−0.37	1.32	−7.5	4.3
19	MTSEK	5–9	20.00	3.81	594	0.71	4.88	−1.9	−0.3
20	FMTSEK	4–9	13.28	5.61	741	2.95	7.40	17.3	12.3
21	IIKNAYKKGE	22–31	15.82	6.89	1162	6.65	8.50	43.1	24.0
22	FKNAIIKNAYKKGE	18–31	15.82	9.87	1622	8.89	8.61	60.2	30.4
23	LVTL	14–17	10.75	7.09	444	5.18	8.15	50.9	24.6
24	SQTPLVTL	10–17	13.73	9.23	857	4.28	6.86	52.4	20.8
25	KSQTPLVTL	9–17	13.13	9.00	985	4.80	6.33	48.7	17.8
26	EKSQTPLVTL	8–17	13.43	9.43	1114	4.73	6.77	41.6	18.9
27	SEKSQTPLVTL	7–17	13.88	9.64	1201	4.17	7.43	37.5	15.7
28	TSEKSQTPLVTL	6–17	14.03	9.42	1302	3.91	6.64	38.3	14.0
29	MTSEKSQTPLVTL	5–17	14.18	9.74	1433	4.99	10.20	43.9	18.0

* Average values determined from regression analysis of the data.

** The summated hydrophobic retention coefficients for individual peptides were calculated according to refs. 34, 35, 36 and 15, respectively.

*** The S and log k_0 value of peptide 18 are not included, due to the very short retention time of this peptide.

whilst the capacity factor at the time of elution, k'_{eff}, (from which most gradient retention coefficients for individual amino acids are derived takes the form

$$k'_{eff} = 1/2.3b \tag{10}$$

Similarly, the value of $\bar{\varphi}$ corresponding to a particular $\bar{k}'$ value can be calculated from gradient data using the expression

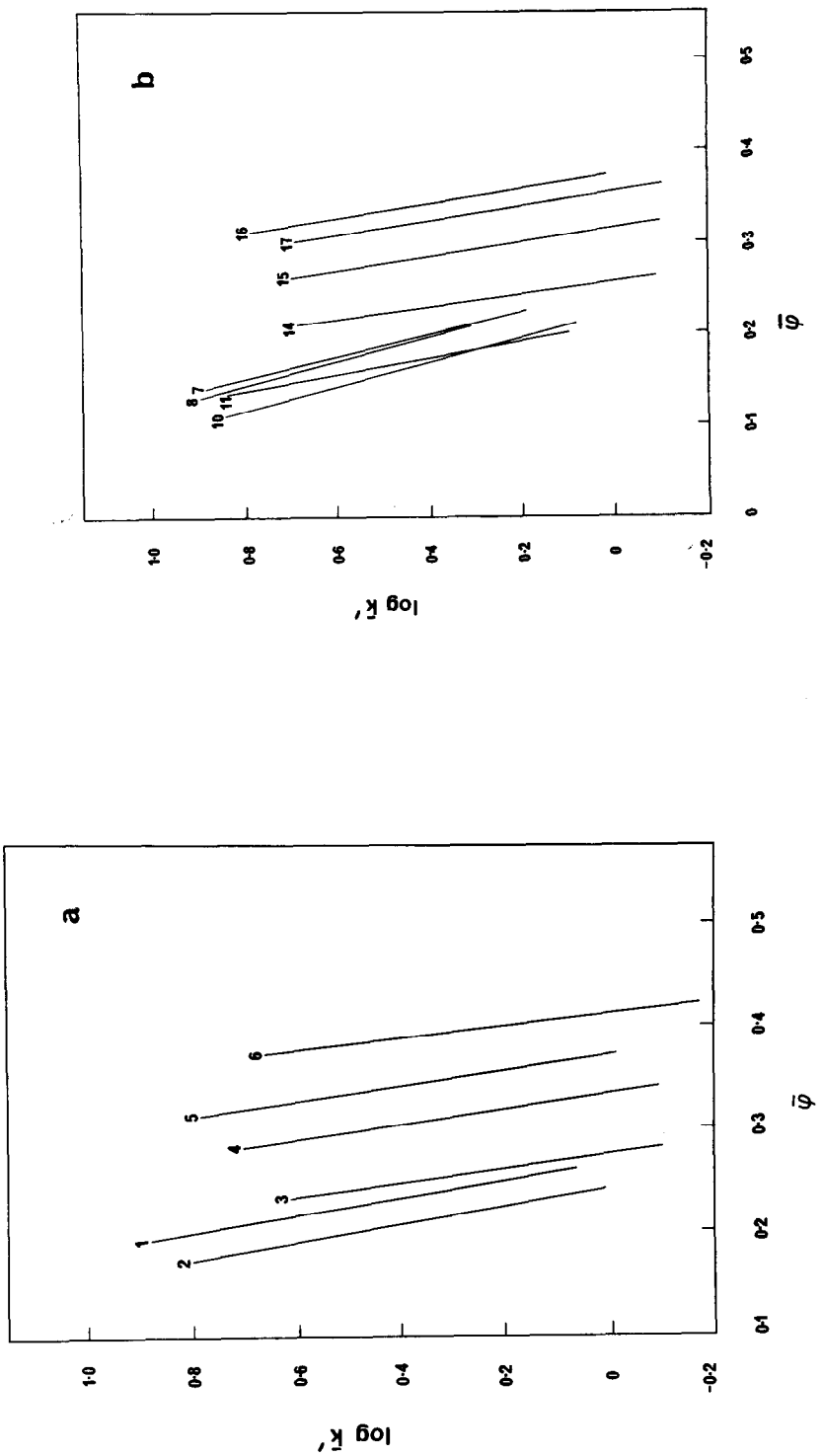
$$\bar{\varphi}_1 = [t_{g1} - t_{0,1} - (t_{0,1}/b) \log 2]/t_{G1} \tag{11}$$

provided k'_0 is large and the gradient duration encompasses 0–100% eluent B.

Consequently, when sufficient retention data have been obtained from gradient experiments, values of $\bar{k}'$ and $\bar{\varphi}$ can be calculated according to eqns. 9 and 11. Since the $\bar{k}'$ and $\bar{\varphi}$ values of different polypeptide components in a complex mixture can be simultaneously determined by using various conditions of gradient time, flow-rate, or $\Delta\varphi$, data acquisition under a wide variety of chromatographic conditions is greatly simplified both in terms of time and experimental flexibility. This in turn enables the isocratic dependencies of k' *versus* φ for numerous polypeptides to be rapidly evaluated directly from the plots of $\bar{k}'$ *versus* $\bar{\varphi}$.

Several corollaries are anticipated from the relationships summarised in eqns. 1–11. Firstly, this treatment suggests that essentially linear dependencies of log $\bar{k}'$ on $\bar{\varphi}$ will occur, provided the solutes are eluted in a regular reversed-phase manner. Secondly, any increase in the magnitude of the gradient steepness parameter, b, will decrease the $\bar{k}'$ (and also the observed final k'_{eff}) value of a polypeptide by a proportional amount. Thirdly, as the V_m of the column is increased, *e.g.*, by using different stationary phases of the same ligand density but more porous particles packed into columns of the same dimensions or alternatively by using the same stationary phase in columns of different configurations, in order to maintain equivalent b values either $\Delta\varphi/dt$ must be decreased or F increased. Fourthly, selectivity differences between two polypeptides will relate to differences in their respective k'_0 and S values as well as to any change in the column V_m value due to different gradient conditions. Thus, changes in band-spacing between polypeptides are anticipated as the S, k'_0, and b parameters are varied, when gradients of different slope or composition are used. Fifthly, from a knowledge of the S, k_w, t_0, b parameters, derived from one gradient condition the predicted t_g values for a particular polypeptide for different t_G, $\Delta\varphi$ and F conditions can be calculated from eqn. 4.

As a initial test of this approach gradient retention data were collected for the 29 β-endorphin-related polypeptides listed in Table I. These polypeptides provide a most useful selection germane to such an evaluation, since they include examples ranging from 4 to 31 residues in length with a molecular weight range of *ca.* 400–3500. Such variations in molecular size and composition are typical of polypeptide mixtures generated from enzymatic digestion or chemical fragmentation of proteins. Further, this selection of polypeptides can be considered to represent five families of peptidic analogues encompassing the N-terminal, C-terminal, and internal spacer regions of a polypeptide hormone of considerable current interest for their different biological functions as well as for their conformational features both in solution and at specific membrane-associated receptors. The chromatographic data concommi-


a
b
log k'
φi
1·0
0·8
0·6
0·4
0·2
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0·1
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0·3
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Fig. 1.

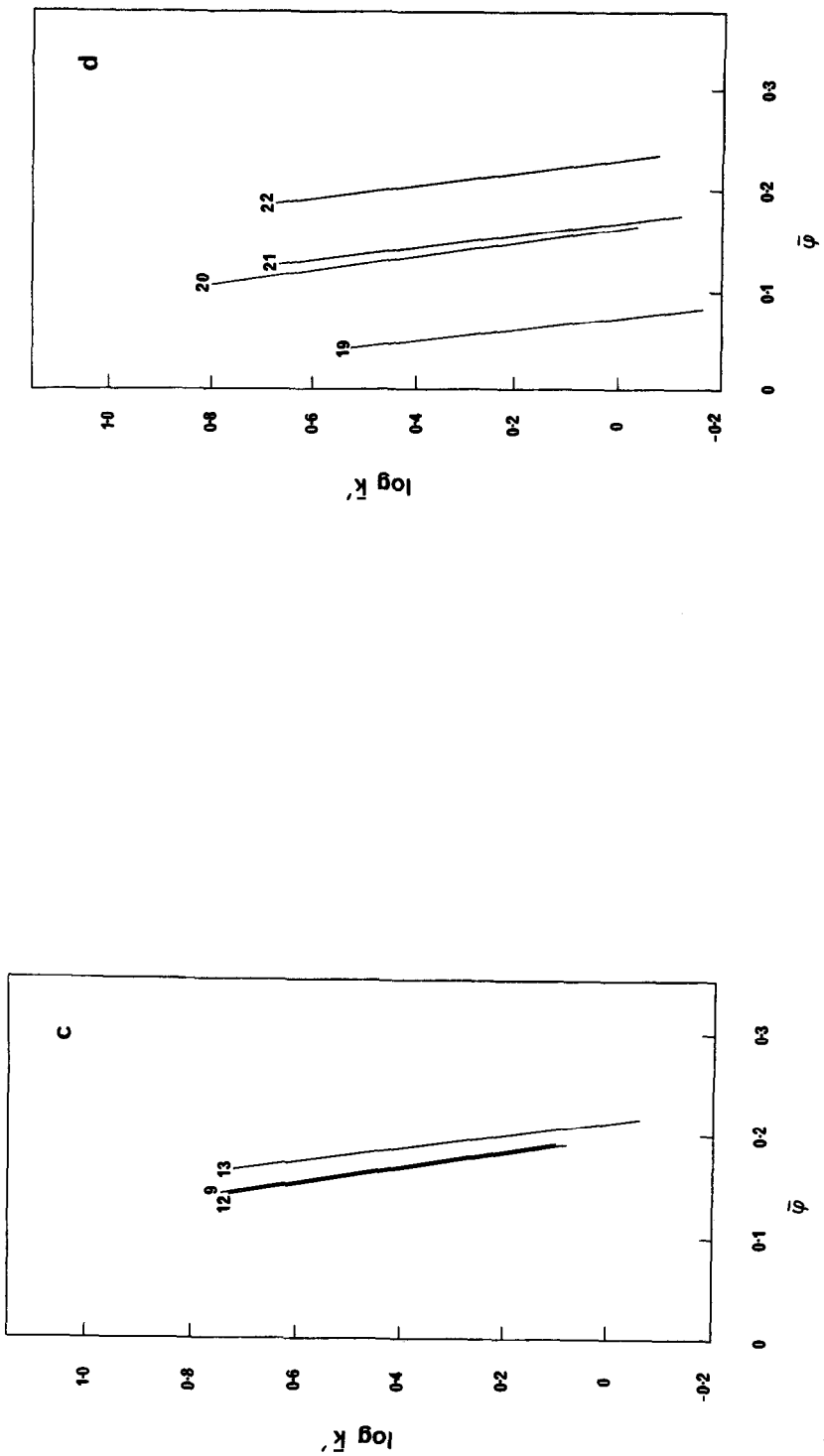

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Fig. 1.

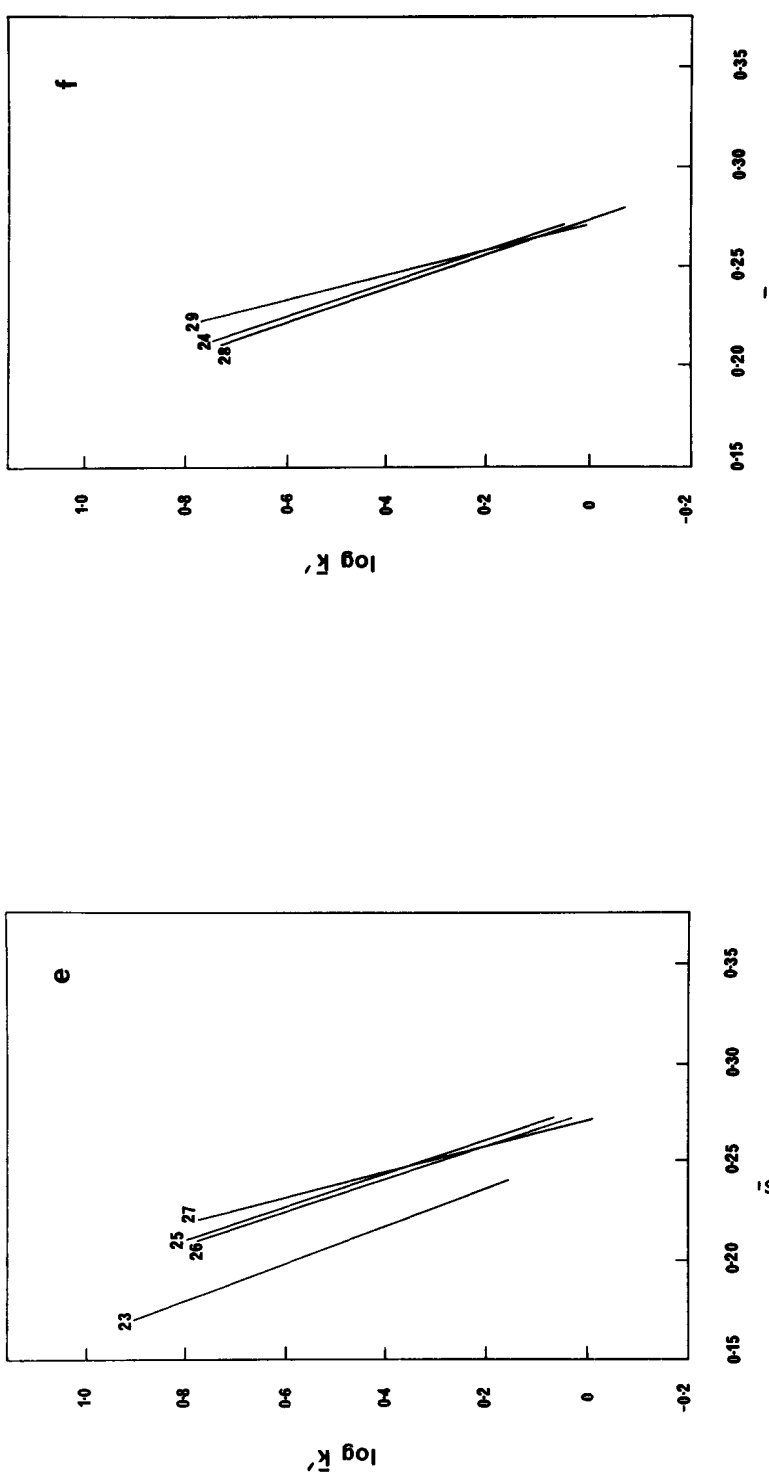


Fig. 1. Plots of k' *versus* $\bar{\varphi}$ based on gradient experiments for β-endorphin (6) and related peptides 1–29. The plots were derived from best fit analysis to the data points obtained from gradient elution experiments, whereby t_G = 20, 30, 40, 60, and 120 min, and F = 1, 2, 3, and 4 ml/min. Other chromatographic conditions are given in the Materials and methods section. See Table I for the code to polypeptide structure and for the calculated slope parameter S and $\log k'_0$ values.

tantly acquired for these various polypeptides thus enables functional group contribution effects to be evaluated and potentially correlated with quantitative structure–activity relationships. In all cases, the gradient data were accumulated with an aqueous eluent containing 0.1% TFA (solvent A) and an aquo-organic eluent containing 50% acetonitrile (v/v) and 0.1% TFA (solvent B). To enable a rigorous evaluation of the gradient retention relationships, gradient times of 20, 30, 40, 60, and 120 min were used at flow-rates of 1, 2, 3, and 4 ml/min. All calculations and regression analyses of retention and resolution parameters were carried out using a composite multivariate programme on a Hewlett-Packard HP86B.

The values of S and log k'_0, calculated from the gradient data for each polypeptide, are listed in Table I. Very close agreement between the gradient elution-derived and the isocratic elution-derived S and log k'_0 values was observed. For example, the values of S for β-endorphin[6] determined from gradient and isocratic data, were 15.1 and 15.7, whilst the corresponding values of log k'_0 were 16.2 and 16.5, respectively. From the numerous gradient experiments (the present study required more than 350 different gradient separations), values of $\bar{k}'$ and $\bar{\varphi}$ were calculated according to eqns. 4–11 for each polypeptide. As is evident from Fig. 1a–f linear dependencies of log $\bar{k}'$ versus $\bar{\varphi}$ were observed for all the 29 polypeptides (the correlation coefficients for the fit of the data of individual peptides to the relationship $\log \bar{k}' = \log k_0 - S\bar{\varphi}$ were between 0.95 and 0.98).

Several features of the gradient (and isocratic) retention behaviour of these β-endorphin-related polypeptides are immediately evident from these figures and the associated data listed in Table I. Firstly, the results indicate that the slopes, S, of the log $\bar{k}'$ *versus* $\bar{\varphi}$ (or the log k' *versus* φ) plots do not follow simple dependencies on the relative hydrophobicities of these peptides, *i.e.*, very low correlations were found with dependencies of the type $S = a\chi + b$, $S = a\chi^b$, $S = ae^{\chi}$ or $S = a + b \ln \chi$ where χ is the relative hydrophobicity as calculated from the summated hydrophobic coefficients[15,34,35] of individual amino acids present in the peptide sequence (Table II). Similarly, no simple relationships were evident between the various $\bar{\varphi}$ values at log $k' = 0$ and the χ values of different polypeptides. From the calculated S values of the β-endorphin polypeptides it is apparent that no simple linear relationship exists between S and molecular weight (MW) (Table II). Using a non-linear least-squares bivariate curve-fitting program, the following approximate relationship ($r^2 = 0.72$) was derived, for these polypeptides and this reversed-phase system:

$$S = 2.99\ (\mathrm{MW})^{0.21}$$

Previously Stadalius *et al.*[13] have derived a similar relationship for model peptides and proteins with molecular weights in the range 600–70 000, *i.e.*

$$S = 0.48\ (\mathrm{MW})^{0.44}$$

Using this alternative relationship for S *versus* MW with the various β-endorphin polypeptides the degree of fit between the experimental and calculated S values was $r^2 = 0.16$. Clearly, as data on a greater variety of peptidic solutes become available more precise definition of the relationship between S and MW will emerge as well as S *versus* molecular sequence and surface topography.

TABLE II

CORRELATION OF THE CALCULATED LOGARITHMIC CAPACITY FACTOR IN NEAT WATER, log k'_0, AND THE SLOPE PARAMETER, S, WITH MW, AND THE SUMMATED HYDROPHOBICITY COEFFICIENTS

The data log k'_0 *versus* MW or log k'_0 *versus* χ were analysed by linear regression methods. Similarly, the data S *versus* MW or S *versus* χ were analysed by linear regression and bivariate curve-fitting procedures. The average values of log k'_0 and S derived from regression analysis of the experimental t_g data were employed.

	r	*Intercept*	*Slope*
log k'_0* *versus*			
MW	0.81	3.61	0.005
χ_1**	0.90	4.80	0.81
χ_2**	0.87	1.62	0.92
χ_3**	0.80	4.51	0.12
χ_4**	0.87	4.18	0.21
S* *versus*			
MW	0.59	11.0	0.002
χ_1**	0.14	12.94	0.07
χ_2**	0.20	12.29	0.13
χ_3**	$1 \cdot 10^{-4}$	13.32	$9.7 \cdot 10^{-4}$
χ_4**	$2.6 \cdot 10^{-3}$	13.55	−0.01
S*** *versus*			
MW	0.72	2.99	0.21
χ_1**	0.03	13.3	−0.01
χ_2**	0.24	10.4	0.12
χ_3**	0.28	9.80	0.08
χ_4**	0.14	11.61	0.04

* Calculated according to equations of the form log $k'_0 = ax + b$ or $S = cx + d$, where x = MW, χ_1, χ_2, χ_3 or χ_4; a, c = slope; b, d = intercept.

** χ_1, χ_2, χ_3 and χ_4 are the summated hydrophobicity retention coefficients for individual peptides, calculated according to χ (or k'_{eff}) = $\Sigma^n C_n \chi_n + d$, using data from refs. 34, 35, 36 and 15, respectively.

*** Calculated according to bivariate analysis of best fit, where $S = 2.99\ (MW)^{0.21}$; $S = 13.3\ (\chi_1)^{-0.01}$; $S = 10.42\ (\chi_2)^{0.12}$; $S = 9.8\ (\chi_3)^{0.08}$; $S = 11.6\ (\chi_4)^{0.04}$. In each case, lower correlations were obtained when the S *versus* χ dependency was expressed as an exponential or logarithmic relationship of the form $S = ae^x$ or $S = a + b \ln \chi$.

Fig. 2 shows the S values for the 29 polypeptides, plotted against molecular weight. The displaced position of peptide[19], MTSEK, is presumably due to error in $\Delta\Delta t_g$, associated with the calculation of the retention data of this very early eluted polar substance rather than anomalous surface interactions with the octadecylsilica stationary phase. Although earlier studies[12–14] have indicated there is an apparent relationship between the magnitude of S and MW for disparate polypeptides of grossly different structure and size, the present results with a well defined set of related polypeptide analogous also suggests that relative hydrophobicity does not play a significant role in determining the magnitude of S. Thus, peptides can be well retained in reversed-phase systems, but exhibit relatively low S values. Such behaviour has been observed with hydrophobic peptide homologues[10,11]. Additional comparison of these data reveals that for many of the β-endorphin peptides the requirements of linear solvent strength gradients have been achieved, *i.e.* similar b values (or equiv-

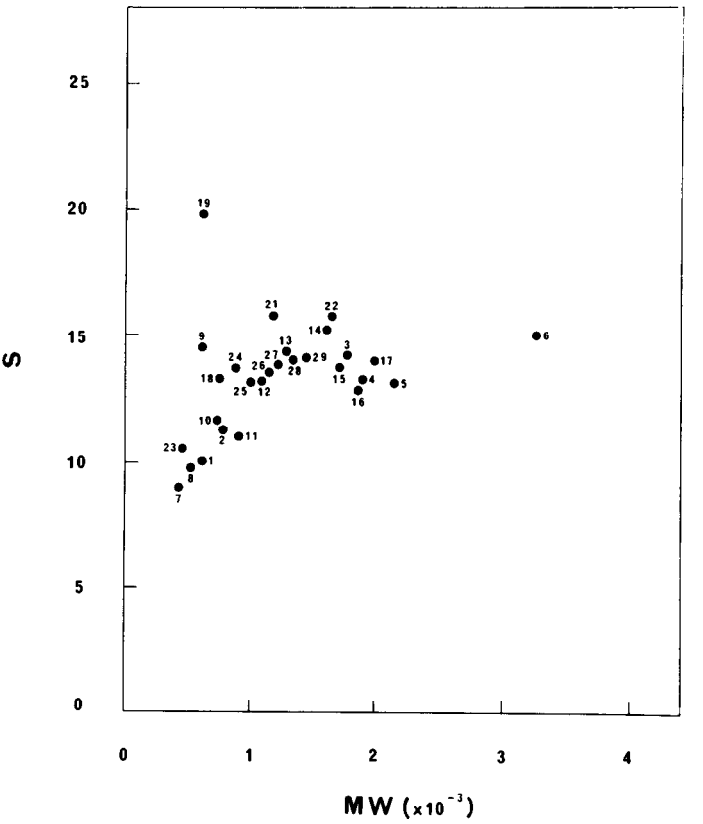


Fig. 2. Relationship between the polypeptide S values and molecular weight for peptides 1–29. See Table I for peptide code and the Materials and methods section for chromatographic conditions.

alent $\bar{k}'$ values at different $\bar{\varphi}$ values) can be obtained for most polypeptides. Illustrating this effect are the plots of the data for $\bar{k}'$ *versus* the summated hydrophobicity coefficients, χ, shown in Fig. 3. Since the variable χ is effectively equivalent to k'_{eff} ($= 1/2.3b$), the results shown in Fig. 3 indicate that chromatographic selectivity has remained essentially constant for many, but not all, of the peptides as t_G was changed from 40 min to 120 min. Further, these results highlight the well known difficulty often faced in attempts to differentiate peptides of different sequence and hydrophobicity on *n*-alkylsilicas by using steep gradients and retention positions calculated from retention coefficients.

It can also be concluded from the data shown in Tables I and II that the log k'_0 values of the various polypeptides follow the order predicted on the basis of the relative hydrophobicities (χ), calculated according to the retention coefficients of Rekker and De Kart[33], Su *et al.*[34], Meek and Rossetti[15] or Browne *et al.*[35]. Further, log k'_0 exhibits an approximately linear dependency on the molecular weight of the solute. Fig. 4 shows the log k_0 data plotted against χ values, derived according to the retention coefficients of Rekker and De Kart. The correlation coefficients for linear regression analysis of log k'_0 *versus* MW, χ_1, χ_2, χ_3, and χ_4 (Table II) were respectively 0.81, 0.90, 0.87, 0.80 and 0.87.

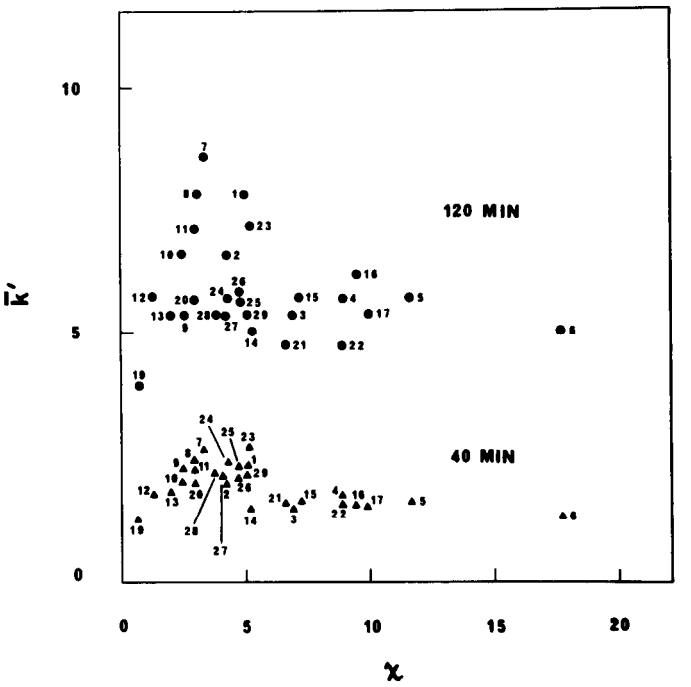


Fig. 3. Plot of $\bar{k}'$ *versus* peptide hydrophobicity (calculated according to ref. 33) for two different gradient times, t_G = 40 and t_G = 120 min. See Table I for peptide code.

As anticipated, the smaller, more polar peptides, *e.g.* peptide 10 or peptide 19, required lower φ values to effect elution compared to the more hydrophobic peptides. Using eqn. 4, values of t_g for the various polypeptides were computed from the t_0, b, k'_0, and t_d values, derived from five different gradient time (*i.e.*, t_G = 20, 30, 40, 60, and 120 min) and different flow-rate experiments. Excellent agreement between the experimentally observed gradient times [t_g(obs)] and the calculated gradient times [t_G(calc)] were obtained from values of log k'_0 and S previously determined by regression analysis. The average standard deviation between the values of t_g(obs) and t_g(calc) for the 29 polypeptides separated under the various gradient conditions of different t_G and F was ± 0.012 (1 standard deviation) in φ units. Greater deviation was evident between the observed and calculated t_g data for the early eluted peptides with gradients of short t_G, *i.e.* t_G = 20 min. This observation is in accord with similar findings[36] for small organic molecules separated under steep gradient conditions by RP-HPLC, particularly in situations where solute pre-elution can occur. Also, the experiments carried out at different flow-rates (varying from 1.0 to 4.0 ml/min) confirmed that both S and log k'_0 values progressively decreased as F increased. This result is consistent with the known curvature of the log k' *versus* φ plots obtained under isocratic conditions. Collectively, these results validate and extend the conclusions reached by Stadalius *et al.*[13] that solute retention times for small molecules and peptidic macromolecules separated by gradient elution RP-HPLC can be accurately and quantitatively represented by eqn. 4.

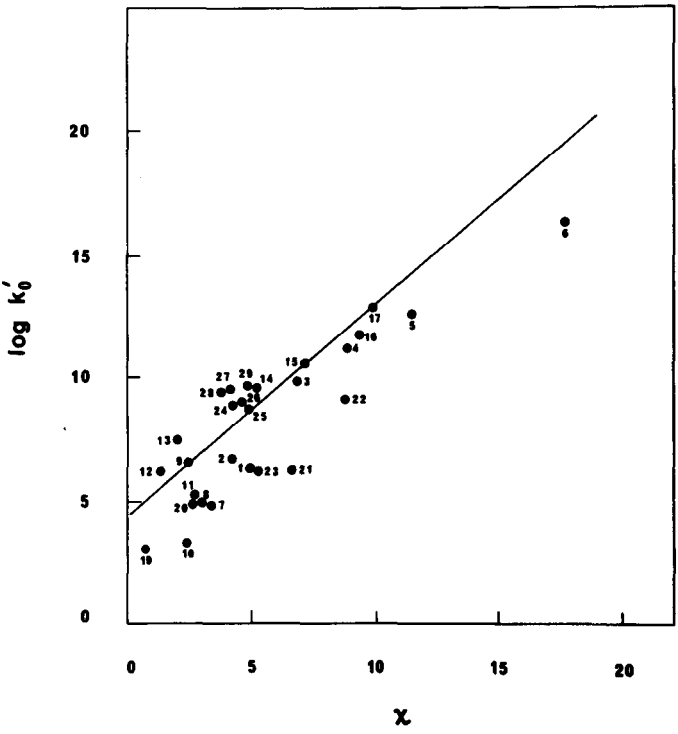


Fig. 4. Plot of log k'_0 *versus* peptide hydrophobicity (calculated according to ref. 33). The solid line corresponds to the best fit to data points (r = 0.90). See Table I for peptide code.

The various data shown in Fig. 1a–f also demonstrate that for polypeptides with different S and log k'_0 values bandspacing can be readily adjusted through appropriate changes in $\bar{k}'$ (or b). This result is of particular relevance to the separation of components which may be unresolved under a particular gradient condition. Typical of this circumstance was the separation of the two polypeptide pairs 9 and 10, and 27 and 28, respectively. The former pair differs only with regard to an additional C-terminal glutamic acid residue (Glu-8) whilst the latter pair differs only with regard to an additional N-terminal threonine residue (Thr-6). Because peptides 9 and 28 have larger S values compared to peptides 10 and 27, respectively, gradient conditions could be developed to resolve these two peptide pairs by progressively decreasing the magnitude of the b parameter by changing F so that different $\bar{k}'$ values were obtained for each polypeptide. As an alternative approach, it is feasible to resolve these pairs by varying the b parameter via appropriate changes in t_G. A similar strategy, utilising this latter approach, has been described by Hartwick *et al.*[37] for the separation of nucleosides under gradient elution conditions (see also refs. 13 and 38).

Finally, the $\bar{k}'$ *versus* $\bar{\varphi}$ data indicate that within the five families of peptide analogues the selectivity remains essentially constant under the different gradient conditions. This observation suggests that quantitative determination of the effects of various common organic modifiers and other mobile phase additives on functional group behaviour can be realised from gradient data. For example, if the resolution of two peptidic analogues, P_i and P_j, under isocratic conditions is considered, the effect of the additional functional group (or groups) on retention can be given by

$$\tau = \Delta \log k'_0 - \Delta S\varphi \tag{13}$$

where

$$\Delta \log k'_0 = \log k'_{0,i} - \log k'_{0,j} \tag{14}$$

and

$$\Delta S = S_i - S_j \tag{15}$$

Since both S and $\log k'_0$ can be estimated for different polypeptides from gradient data, the conditional dependence of τ on experimental variables and solute structure can be readily evaluated. For example, by using organic solvent normalisation approaches[25,39], the effect of different organic modifiers on peptide selectivity and retention can thus be predicted. More importantly, this treatment also allows the effect of changes in organic solvent concentration on structural elements of a polypeptide to be assessed. In view of the importance of τ values in quantitative structure–activity relationships for pharmaceutical compounds, the prospect that RP-HPLC methods similar to those described above can be developed for biologically active polypeptides is particularly appealing. For example, β-endorphin (peptide 6) shows[40] significantly higher opiate activity than does α-endorphin (which encompasses the first 16 residues of β-endorphin *i.e.* peptide 3), although the C-terminal region of β-endorphin does not appear to have any highly specific function in binding to the μ- or δ-opiate receptors or in the guinea pig ileum activity assay. Secondary structure predictions indicate that the carboxyl terminal half of β-endorphin shows a high helical potential in the presence of lipids at low pH, whilst α-endorphin has a low helical profile in water at neutral or acidic pH values or in the presence of lipids. These predictions are in accord with the observation[41] that β-endorphin, but not α-endorphin, undergoes a decrease in molecular volume when chromatographed in SEC systems in the presence of acidic lipids. It has been concluded from this and other recent studies with peptide analogues that peptides which bind to amphiphilic surfaces, such as membranes, receptors or phospholipid vescicles probably possess regions of amphiphilic secondary structure complementary to those of the target surfaces. Blanc and Kaiser[42], and Hearn[43] have proposed that such an amphiphilic α- or π-helical region is capable of forming between residues Pro-13 and Gly-30 of β-endorphin. This amphiphilic element is separated from the highly specific N-terminal region opiate recognition site corresponding to the first five residues (YGGFM) by a hydrophilic leash region between residues Thr-6 and Thr-12. The hydrophobic domain resulting from the formation of this helical structure appears to be of critical importance in the specific interaction of β-endorphin with the ε-receptor and probably strongly influences peptide peptide lipid interactions. As reported elsewhere[44], studies of τ *versus* φ for β-endorphin and other polypeptide hormones have provided a basis for quantifying the effect of organic modifier type and concentration on group behaviour via linear free energy relationships. Further, evaluation of these τ dependencies should allow constitutional and environmental factors which affect polypeptide structure, including the formation of amphiphilic elements, at lipid surfaces, to be discriminated.

Resolution relationships in gradient elution

Previous experimental studies[8,13,45] with peptides have demonstrated that both the peak capacity, PC, and the peak height are inversely related to the gradient steepness parameter. Since the average resolution, R_s, for the separation of a complex mixture of polypeptides under a particular gradient condition can be equated with the peak capacity, determination of peak capacities under different chromatographic conditions of t_G, F, V_m, etc., provides a basis for optimising the resolving power of the system and also reveals important information on anomalous retention and peak shape relationships unique to some polypeptides. The peak capacity for a chromatographic separation of gradient time, t_G, and resolution, $R_s = 1$, for all adjacent zone pairs can be defined as

$$PC = t_G/4\sigma_t \tag{16}$$

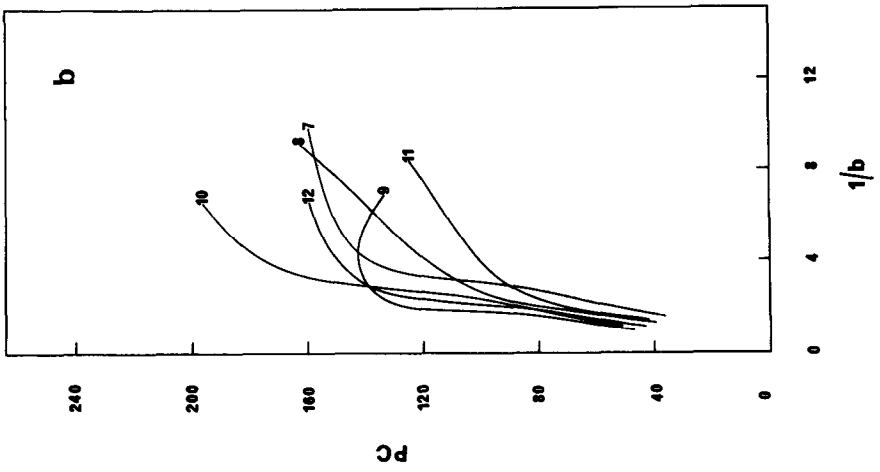
where σ_t is the average bandwidth in time units of the various elution zones. As the b value becomes larger (*i.e.* steeper gradients) the bandwidth of all eluted zones becomes narrower. As Snyder has shown[30], the peak capacity can be given by

$$PC = (2.3/4)\,\Delta\varphi/(2.3b + 1)G \tag{17}$$

where G is the band compression factor due to the increase in solvent strength across the solute zone as it migrates through the column. The band compression factor is related to the bandwidth, σ_t, the gradient steepness parameter, b, and the plate number, $\bar{N}$, for polypeptides with large k'_w values through the expression

$$\sigma_t = Gt_0(2.3b + 1)/2.3b\bar{N}^{1/2} \tag{18}$$

It can be seen from eqns. 17 and 18 that peak capacity will depend not only on such experimental variables as gradient slope, $\Delta\varphi$ range or column t_0 value but also on how plate number and peak variance change with the chromatographic conditions and solute structure. If b is fixed then changes in peak capacity will directly reflect variation in plate height. Furthermore, eqns. 17 and 18 predict that the peak capacities for a series of polypeptides, separated under a given set of chromatographic conditions, will show asymptotic dependencies on b. Furthermore, over a defined k' and $\Delta\varphi$ range, peak capacities should increase linearly with S, provided that σ_t (or σ_v) of early and late eluted compounds remain the same. The experimental results obtained in the present and earlier[8,13,15] studies confirm the general form of the dependency of PC on b. Typical results of the change in peak capacity of individual polypeptides, plotted as a function of $1/b$ are shown in Fig. 5a–f. These data clearly indicate the advantages with very complex mixtures of polypeptides to use relatively large $\Delta\varphi$ values in gradient elution ($\Delta\varphi = 0.5$ was used in the present study; a relatively common selection) to generate acceptable resolution. With polypeptides which exhibit similar retention times great care in the selection of the appropriate $\Delta\varphi$ range, t_G and F is essential if high peak capacities are to be achieved. Further, the changing pattern of PC *versus* $1/b$ behaviour seen with these polypeptides reflects the composite effects of molecular size and relative hydrophobicity on PC. For example, the resolution of larger polypeptides, *e.g.* peptides 6, 17 or 22 was generally characterised by peak capacities which were smaller than those observed for small polar peptides, *e.g.*, peptides 1, 2 or 19, at comparable b values. However, much


b
pC
1/b
0
40
80
120
160
200
240
0
4
8
12
7
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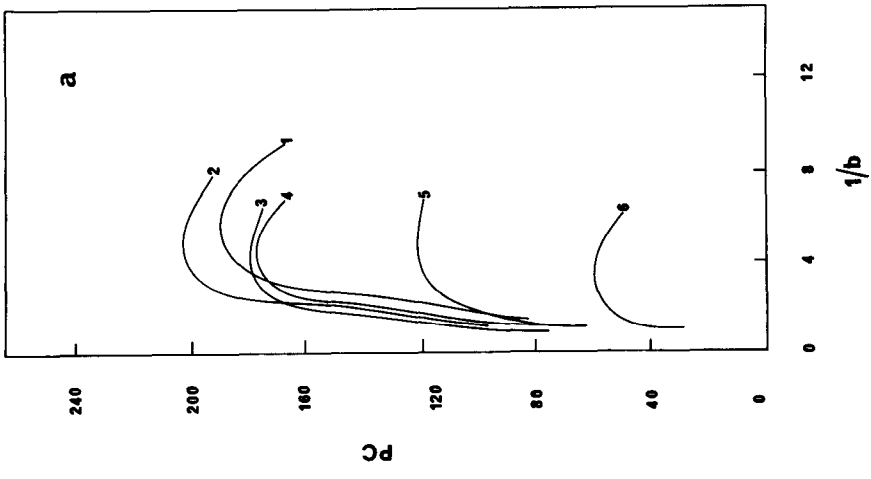
11
12


a
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0
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Fig. 5.

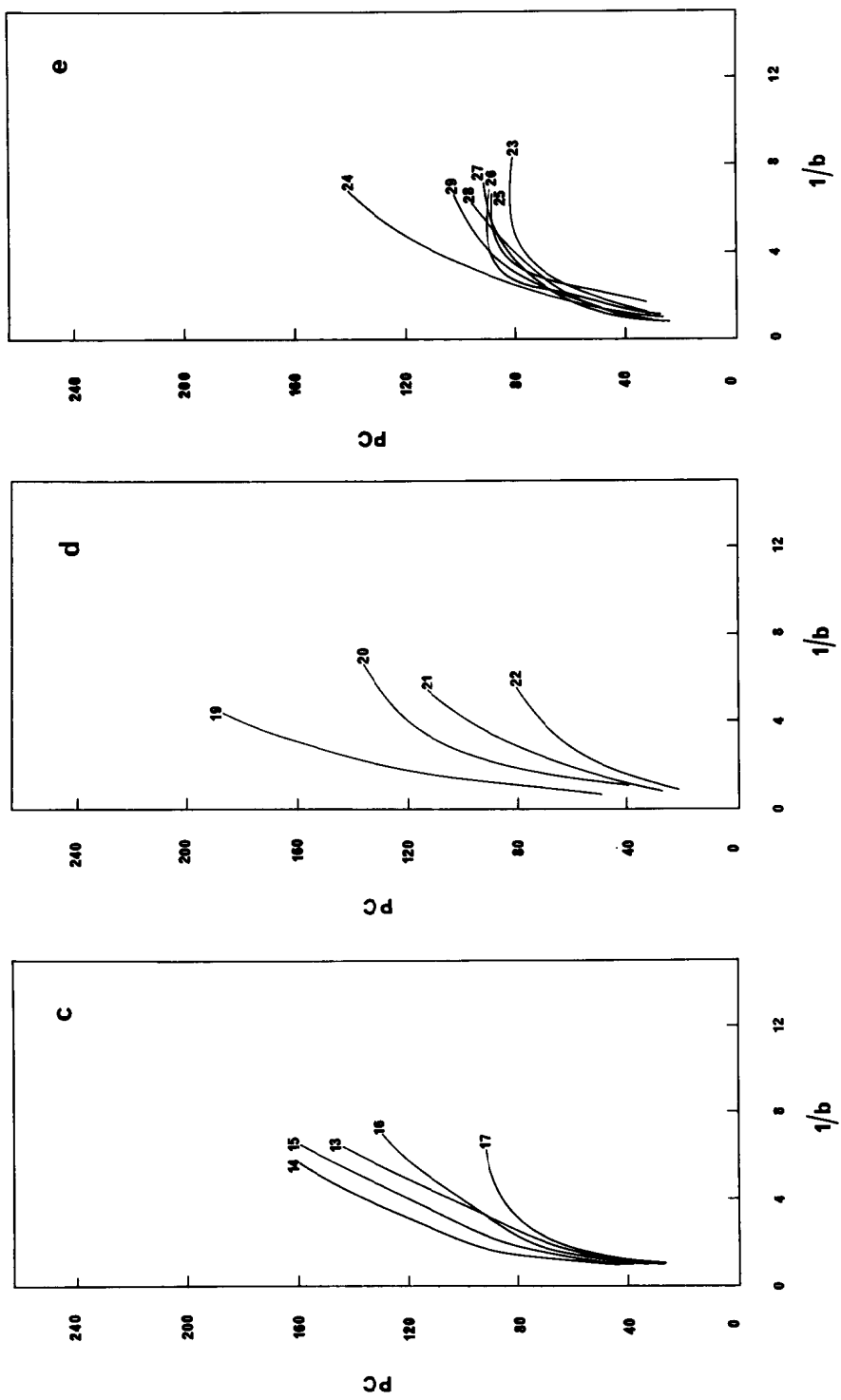

Fig. 5. Plots demonstrating the relationship between peak capacity, PC, and the gradient steepness parameter, b, for the β-endorphin-related polypeptides 1–29. The gradient slope and/or flow-rate were adjusted as appropriate to each experiment. See Table I for peptide code.

more importantly, these investigations demonstrate that other factors besides the expected retention parameters (S, k'_0, $\Delta\varphi$, $\bar{k}'$) influence PC. Although changes in several experimental variables ($\Delta\varphi$, t_G, F, t_0) affect b values and hence retention, manipulation of these variables also affects peak widths. The changes in shape and curvature evident in the plots of peak capacity *versus* $1/b$ thus reflect the composite effects of these variables on solute retention and kinetic processes. Comparison of the peak capacity data for several polypeptides are, in this context, illustrative. Thus, the maximum peak capacity calculated for the tetrapeptide LVTL (peptide 23, MW 444, $S = 10.8$, $\log k'_0 = 7.1$) was *ca.* 80 and similar to that found for much larger polypeptides, *e.g.*, GGFMTSEKSQTPLVTLFK (peptide 17, MW 1969, $S = 14.0$, $\log k'_w = 9.9$) at comparable, b, $\bar{k}'$, or $\bar{\varphi}$ values. What then is the origin of these differences in peak capacities seen with these β-endorphin-related polypeptides as the gradient steepness is changed? Some clue to the mechanisms by which σ_v of individual polypeptides can change under different t_G, $\Delta\rho$, and F conditions has been obtained from a detailed evaluation of peakwidth and band asymmetry (a_s) data, given in an accompanying paper[45]. These data on σ_v and a_s for the β-endorphin-related polypeptides and other hormonal peptides have indicated that some peptides exhibit unexpectedly large bandwidths and anomalous asymmetry behaviour, depending on the gradient duration, the dwell or residence time the peptide spends on the stationary phase surface, the temperature, and the mobile phase composition. Typically, these peptides contain elongated linear regions of hydrophobic residues, sequence elements capable of forming amphiphilic helical secondary structures or proline-rich peptides. Similar anomalous behaviour, associated with an apparent decrease in resolving power of an otherwise highly efficient gradient or isocratic system, has already been documented[9,28,29] with proline dipeptides and some proteins. Since maximum peak capacity will occur with a specified column and elution condition (t_g, F, $\Delta\varphi$) when a minimum average bandwidth is achieved (*cf.* eqns. 16 and 20), any kinetic phenomena which lead to gross changes in σ_v and a_s will result in decreased PC or average resolution. Investigations currently underway should resolve the nature of some of these kinetic processes and their involvement in peak capacity differences typified by the behaviour of peptide 23.

SYMBOLS

a_s	Peak asymmetry factor
b	Gradient steepness parameter as defined by eqn. 5
C	Intercept variable as derived from plots of log k' *versus* [solvent] at [solvent] = 0
D	Slope (or tangent) of the plot log k' *versus* φ over the silanophilic range of retention behaviour
F_1, F_2 ...	Flow-rate of mobile phase at different values
G	Band compression factor as defined by eqn. 18
k'_i	Capacity factor for solute P_i determined from isocratic retention data
$\bar{k}'_i$	Median capacity factor for solute P_i determined from gradient retention data as defined by eqn. 9
k'_0	Capacity factor for solute P_i determined under isocratic conditions at $\varphi = 0$

$\bar{k}'_0$	Median capacity factor for solute P_i derived at $\varphi = 0$
k'_P	Capacity factor for solute P_i determined under isocratic conditions at $\varphi = 1.0$
m	Slope (or tangent) of the plot log k' *versus* [solvent] as defined by eqn. 2
MW	Molecular weight
$\bar{N}$	Median theoretical plate number as defined by eqn. 18
PC	Peak capacity as defined by eqn. 16
r	Correlation coefficient
R_s	Average resolution as defined by eqn. 8
S	Slope (or tangent) of the plot log k' *versus* φ or log k' *versus* φ as defined by eqn. 1
t_d	Chromatographic dwell time
t_{g1}, t_{g2} ...	Gradient elution times for solute P_i under two different gradient conditions
t_{G1}, t_{G2} ...	Gradient times
$t_{0,1}$, $t_{0,2}$ ...	Column dead times for different conditions of flow, etc.
t_{SEC}	Retention time for the SEC component
$V_{g,1}$, $V_{g,2}$ ...	Gradient elution volumes for solute P_i under different gradient conditions
V_m	Mobile phase volume
$V_{0,1}$, $V_{0,2}$	Column dead volumes for different conditions of flow, etc.
$\bar{\alpha}_g$	Gradient selectivity factor as defined by $\alpha_g = k'_i/k'_j$
β	Ratio of gradient times as defined by $\beta = t_{G1}/t_{G2}$
$\Delta\varphi$	Change in organic solvent modifier mole fraction
$\Delta \log k'$	Difference in logarithmic capacity factors at $\varphi = 0$ as derived from eqn. 14
ΔS	Difference in slopes, S_i and S_j, for two solutes P_i and P_j
$\Delta\Delta t_g$	Error in measurement of difference in two gradient retention times
ρ_p, ρ_r, ρ_s	Mole fractions of solute associated with silanophilic, solvophobic and size-exclusion retention behaviour
σ_t	Peak bandwidth in time units
σ_v	Peak bandwidth in volume units
χ	Hydrophobic retention coefficient
θ'	Rate of change of organic modifier with time ($\theta' = \Delta\varphi/t_G$)
φ	Organic solvent modifier mole fraction in isocratic system
$\bar{\varphi}$	Median organic solvent modifier mole fraction as defined by eqn. 11
τ	Solute selectivity parameter ($= \ln \alpha$)

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