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

XXVII. SOLVOPHOBIC CONSIDERATIONS FOR THE SEPARATION OF UNPROTECTED PEPTIDES ON CHEMICALLY BONDED HYDROCARBONACEOUS STATIONARY PHASES*

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SUMMARY

Methods for the manipulation of peptide retention and selectivities on hydrocarbonaceous microparticulate silicas are discussed. In particular the influence of the mobile phase water content, ionic strength and pairing ions on the retention behaviour of peptides and polypeptides on octadecylsilicas has been examined. Under these changing elution conditions with reversed-phase systems, peptide retention can be rationalised in terms of solvophobic theory. This treatment provides a valuable predictive approach for evaluating the distribution equilibria involved in the interaction of peptidic solutes with the mobile and stationary phases.

INTRODUCTION

Over the past several years, reversed-phase high-performance liquid chromatography (HPLC) has become a versatile technique for resolving mixtures of peptides. In recent publications, we have reported[1–6] the development of a number of elution strategies which have allowed the reversed-phase HPLC analysis, and in selected cases the isolation, of a variety of natural and synthetic peptides including insulin-related peptides, pituitary peptides and polypeptides, as well as peptides from chemical or enzymatic digestion of proteins. It was apparent from these, and other studies[7–12], that separation selectivities for unprotected peptides chromatographed on hydrocarbonaceous silicas can be manipulated by a number of mobile phase parameters including the mobile phase water content, the type of organic solvent modifier, the ionic strength, the pH, the temperature, the concentration and the chemical nature of eluent components which can engage in ion-pair formation or dynamic liquid–liquid ion-exchange interactions. Additional control over retention can be achieved

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by appropriate changes in the hydrophobicity of the stationary phase, *e.g.* by variation in the carbon loading and chain length of the alkyl-ligand.

It is well recognised that column selectivities in the reversed-phase HPLC separation of peptides arise due to differences in the distribution equilibria established by the various peptide solutes between the stationary and the mobile phase and thus peptide retention will be governed by thermodynamic criteria. The aim of this investigation was to study the retention behaviour of a group of peptides and polypeptide hormones in a reversed-phase system with an octadecylsilica stationary phase under conditions where the mobile phase water content or the ionic strength were varied and in circumstances where ion-pairing or dynamic ion-exchange events can occur. A semi-empirical approach, based on solvophobic considerations, has been developed to rationalize solute retention and used in the analysis of selectivity factor differences for peptides separated by reversed-phase HPLC.

EXPERIMENTAL

Chemicals and reagents

All solvents were AnalaR grade, acetonitrile, methanol and water were purified as described earlier[2]. The source of some of the peptides and polypeptides (Table I) used in this study has been given previously[1,2], the remainder were either prepared and purified in this laboratory or purchased from Sigma (St. Louis, MO, U.S.A), Bachem (Torrance, CA, U.S.A.), Research Plus Labs. (Denville, NJ, U.S.A.) or Calbiochem (La Jolla, CA, U.S.A.). The β-Met- and β-Leu-endorphins were generously provided by Drs. J. Rivier (Salk Institute) and D. Coy (Tulane University). All amino acids except glycine, were of the L-configuration. D-Camphor-10-sulphonic acid was obtained from BDH (Poole, Great Britain), sodium hexane sulphonate was prepared from the corresponding alkylbromide[13]. Orthophosphoric acid, sodium hydroxide and sodium dihydrogen phosphate were obtained from May and Baker (Dagenham, Great Britain).

Apparatus

All the 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 440 fixed wavelength (254 nm) or a Model 450 variable-wavelength UV detector, all from Waters Assoc., (Milford, MA, U.S.A.) and a Rikadenki dual-channel recorder. Sample injections were made with Pressure-Lok liquid syringes (0–25 μl) series B110 from Precision Sampling (Baton Rouge, LA, U.S.A.). The pH measurements were performed with a Radiometer PHM64 Research pH meter, equipped with a Radiometer combination electrode.

Methods

All chromatograms were carried out at ambient temperature (*ca.* 20°C). All peptides were made up in the eluting solvent. Bulk solvents were degassed separately and the appropriate mobile phases prepared and equilibrated to operating conditions as reported previously[2]. All columns were equilibrated to new mobile phase systems for at least 30 min (*ca.* 50–100 column volumes). Sample sizes varied between 10 and 100 μg of peptide material injected in volumes of 5–25 μl. The relative capacity factors

TABLE I

LINEAR SEQUENCES OF PEPTIDES AND POLYPEPTIDES USED IN THE PRESENT STUDY

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

No.	*Peptide*	*No.*	*Peptide*
1	G	26	YGGFM (Met-enkephalin)
2	GG	27	CYIQNCPLG (oxytocin)
3	GGG	28	SYSMEHFRWGKPVGKKRRPVKVYP
4	GV		($ACTH_{1-24}$)
5	GF amide	29	YGGFL (Leu-enkephalin)
6	GG ethyl ester	30	GIVEQCCASVCSLYQLENYCN
7	GGY amide		(bovine insulin A chain)
8	GLA	31	DRVYIHPF (angiotensin II)
9	GLY	32	SYSMEHFRWGKPV (α-MSH)
10	GGGA amide	33	DRVYIHPFHL (angiotensin I)
11	AG	34	RPKPQQFFGLM (substance P)
12	FF	35	RPVKVYPNGAEDESAEAFPLEF
13	FFFF		(CLIP)
14	FA	36	AGCKNFFWKTFTSC
15	FL amide		(Somatostatin)
16	KG	37	Bovine insulin
17	RF	38	FVNQHLCGSHLVEALYLVCGERGFF
18	RFA		YTPKA (bovine insulin B chain)
19	MRF	39	YGGFMTSEKSQTPLVTLFKNAII
20	MRFA		KNAHKKGQ (β-Met-endorphin)
21	LW	40	YGGFLTSEKSGTPLVTLFKNAII
22	LWMR		KNAHKKGQ (β-Leu-endorphin)
23	LWM		
24	LWMRF		
25	CYFQNCPKG (lysopressin)		

for isocratic and gradient experiments were calculated and the data analysed as reported previously[1,21]. The low pH conditions of pH 2.3–3.0 were chosen on the basis of criteria established in our earlier studies[1,4] to ensure adequate control over protonic equilibria. The pH was adjusted where necessary with 10 *M* NaOH. Flow-rates were generally 2.0 ml/min for the isocratic elution experiments and 1.0 ml/min for the gradient elution experiments.

RESULTS AND DISCUSSION

Theoretical considerations

In the present basic model for the separation of peptides and related ionogenic solutes, on non-polar stationary phases, we assume that reversible interactions occur between the peptide molecules S_1, S_2, ... S_n and the hydrocarbonaceous ligand L, and that these interactions are due to hydrophobic associations and not to electrostatic or hydrogen bonding effects. Depending on whether the bonded hydrocarbonaceous ligand effectively acts as a bulk liquid involving solvophobic aggregates of non-polar liquid droplet clusters or as a relatively ordered, uniformly adsorptive monolayer of isolated alkyl "bristles", the sorption process can be described[14,15] in

terms of partition or adsorption events. In the partition process the retention of the solute, S_i is related to the phase volume via the partition coefficient, P_i, whilst in an adsorption process, solute retention is related to the total interfacial surface area via the adsorption coefficent, $K_{A,i}$. Solute retention for a series of peptides will thus be characterised by a set of equilibrium constants K_1, K_2, ... K_n such that, in the general case, the capacity factor for the peptide solute, S_i will be given by

$$k_i' = \varphi \cdot K_i \tag{1}$$

where

$$K_i = \frac{[S_iL]}{[S_i]\,[L]} \tag{2}$$

and φ is the phase ratio in the column.

Since the equilibrium constants are related to the overall standard unitary free energy changes associated with the transfer of the peptide solutes from the mobile to the stationary phase, then solute retention can be expressed as

$$\log k_i' = \log \varphi - \frac{\Delta G_{assoc,i}}{RT} \tag{3}$$

where R is the gas constant and T is the absolute temperature.

Chromatographic selectivity, $\alpha_{i,j}$, for two peptides, S_i and S_j, can thus be given by

$$RT \log \alpha_{i,j} = \Delta(\Delta G^0_{assoc,i,j}) \tag{4}$$

Because the physico-chemical basis of classical liquid–liquid partition chromatography and chromatographic separations of peptides on hydrocarbonaceous silicas is similar, it is also possible to express selectivities in terms of partition coefficients such that

$$\log \alpha_{i,j} = \log P_i/P_j \tag{5}$$

where P_i and P_j are the partition coefficients of the peptides S_i, S_j respectively for a particular stationary–mobile phase combination. The $\log \alpha_{i,j}$ term thus reflects the retention characteristic properties of two peptides, S_i and S_j, which differ by a functional group and is analogous to the ΔR_M term[16,17] used to predict selectivity differences in thin-layer chromatographic separation of peptides as well as other extra-thermodynamic terms such as the Hansch π terms[18], the Rekker hydrophobicity fragmental constants f[19], or the free energy of transfer, ΔG_t, terms for amino acid side chains as proposed by Nozaki and Tanford[20].

Based on the above free energy relationships, selectivity parameters can be related to the organic solvent concentration by

$$\log \alpha_{i,j} = a[\text{organic modifier}] + b \tag{6}$$

and empirically to topological indices, the general form of this equation being

$$\log \alpha_{i,j} = c\chi_{i,j} + d \tag{7}$$

where $\chi_{i,j}$ is an appropriate liquid–liquid group distribution parameter. Both log $\alpha_{i,j}$ and log P_i/P_j can thus be readily derived for the solutes S_i and S_j with a particular stationary mobile phase combination, and in circumstances where P_i and P_j are available, *e.g.* when computed from topological indices or from liquid–liquid partition experiments, selectivity differences may be predicted and the experimentally determined data analysed with respect to changes in the mobile phase composition, temperature, etc. For example, the effect of different isocratic elution systems, *m* and *n*, on the retention of S_i with a fixed stationary phase can be interrelated[21] by

$$\log k'_{i,m} = z \log k'_{i,n} + e \tag{8}$$

where $k'_{i,m}$, $k'_{i,n}$ are the capacity factors of S_i in the mobile phases *m*, *n* respectively, *z* is the relative elutropic strength parameter and *e* a system constant.

In order to evaluate precisely the influence of the mobile phase characteristics on the k' values of peptides separated on a particular type of non-polar stationary phase, a solution to eqn. 3 is required. Solvophobic theory, as advanced by Sinanoglu and co-workers[22,23], has been successfully adapted by Horváth *et al.*[24] to permit an evaluation of ΔG^0_{assoc} for the reversed-phase separation of neutral substances. This theoretical approach has revealed that the capacity factor is a function of a number of parameters including the interfacial surface tension γ; the relative surface area, ΔA of the solute molecule in contact with the stationary phase which can be indicated by the molecular surface area, A; the static dipole moment, μ, of the solute molecule; the polarisability of the solute molecule; the static dielectric constant, ε, of the solvent; and the microscopic cavity factor, κ^c, which may be defined as the ratio of the energy required to create a cavity for a solvent molecule to the energy required to extend the planar surface area of the solvent by the surface area of the added solute molecule. The interfacial surface tension between the mobile and stationary phases is reflected in the partitional properties of the two phases (and hence can be expressed in terms of log $\alpha_{i,j}$, log P_i/P_j and related solubility parameters[25]). The relationship between the capacity factor, k', for unionised solutes, weak organic acids and bases separated on reversed phases, and the eluent surface tension γ for aquo–methanol and aquo–acetonitrile mobile phases may be given[24] by

$$\log k'_i = C + \gamma \frac{N\Delta A_i + 4.836\, N^{1/3} (\kappa_i^c - 1)\, V_m^{2/3}}{RT} \tag{9}$$

where C is a constant, and N, V_m, R and T are Avogadro's number, the average molar volume of the mobile phase, the gas constant and the absolute temperature, respectively.

If the retention of peptides on alkyl-silicas is based on a similar sorption mechanism, then a linear dependency of log k'_i *versus* γ for peptide S_i is anticipated. Changes in the water content of the mobile phase are known[2,4,8,9] to elicit dramatic effects on peptide retention on reversed phases and this feature can be usefully exploited in gradient elution. It also follows that for linear gradients commonly used in peptide separation (*e.g.* water–0.1% orthophosphoric acid with a 0.25–0.5% per

min acetonitrile or methanol modifier[1,2,6] where the apparent capacity factor, $k'_{i,app}$ can be found by[26]

$$\log k'_{i,app} = (\log k'_{i,w} - \omega\psi_0) - \omega \frac{d\psi}{dt} \cdot t \qquad (10)$$

where $k'_{i,w}$ is the isocratic k' value of S_i in pure water, ψ_0 the volume fraction of the beginning of the gradient and the coefficient, ω, is related to the elutropic strength of the pure organic solvent modifier), quasi-linear relationships should exist between $k'_{i,app}$ and ψ and between $\log k'_{i,app}$ and γ for a series of peptides S_1, S_2, ... S_n. A given peptide will elute at characteristic values of ψ and γ for a particular stationary–mobile phase system. From eqn. 9, we can also see that for a homologous series of peptides in which amino acid residues are added in an ordered manner, plots of $\log k'$ *versus* number of residues should be linear, *i.e.* $\Delta(\Delta A)$ is constant, whilst for peptides of different composition, for positional isomers of the same composition, or for diastereoisomers, the k's should follow changes in the effective hydrophobic contact area, ΔA. Clearly, for positional isomers ΔA differences will depend on the relationship of hydrophobic side chains to ionised centres and on the extent of peptide folding whilst for diastereoisomers, ΔA differences can be based on stereochemical arrangements in which the side chains bear cisoid or transoid relationships to each other. Retention data reported in several studies[4,9,12] are consistent with these conclusions and similar trends were evident with the present series of peptides examined. In accordance with previous studies[6,9,12,27], glycine and its oligomers were found to show little appreciable retention to octadecylsilicas over the pH range 2.1–7.5 with phosphate buffers and only relatively small k' values with buffers containing alkyl-sulphonates or -sulphates. These results suggest that the peptide chain proper probably makes only a very small contribution to the retention process for peptides on reversed phases under low pH elution conditions. With simple aquo–organic solvent mobile phases at low pH values, the selectivity differences and k' values for the peptides and polypeptides listed in Table I were, in general, dependent on the summated effect of the relative hydrophobicities[19,29] of the different amino acid side chains present in the various peptides with the position of the side chains in relation to charged sites playing an important, but secondary, role. Similar conclusions can be drawn from a number of other studies, including the separation of peptide positional isomers[6,11,12] and diastereoisomers[12,28] by reversed-phase HPLC.

As indicated above, it should be possible to estimate the elution order for peptides on reversed phases under isocratic and linear gradient conditions using suitable topological indices which take into account the effective hydrophobic contribution which each of the amino acid side chains make to the retention process. Table II shows the data for the experimentally observed k' (apparent) for the peptides (17–24) on a μBondapak C_{18} column using a linear 0.83 % per min acetonitrile gradient and values computed by linear regression for two independently derived sets of hydrophobic retention indices as developed by Rekker[19] and Meek[29], respectively. As can be seen by comparison of the observed and predicted elution order for this group of peptides, both sets of indices gave reasonable correlation although some discrepancies can be noted (*e.g.* the observed elution order of peptides 15 and 16 is opposite to that predicted by the retention coefficient approach[29]). Similar regional

TABLE II

OBSERVED AND PREDICTED APPARENT CAPACITY FACTORS FOR THE PEPTIDES 17–24

Peptide	k'_{appar}*	k'_{pred}**	$\Delta k'$	k'_{pred}***	$\Delta k'$
17	1.71	2.08	+0.37	2.35	0.64
18	2.29	2.47	+0.18	2.33	0.04
19	4.64	2.95	−1.69	3.70	−0.66
20	4.94	3.50	−1.44	3.70	−1.24
21	5.41	5.80	+0.41	5.96	0.55
22	5.76	5.76	0.0	6.46	0.70
23	6.94	8.24	+1.30	7.33	0.39
24	9.00	12.00	+3.00	9.14	0.14

* Column: μBondapak C_{18}; flow-rate, 1 ml/min; a 60-min linear gradient was generated from water–0.1% H_3PO_4 to 50% acetonitrile–50% water–0.1% H_3PO_4.

** Calculated by linear regression from the summated hydrophobic fragmental constants for the amino acid side chains according to refs. 19, 21 and eqns. 5–7 which assumes log k'(peptide) = $a'\Sigma$f (amino acids) + b' and using Σf(amino acid) values from ref. 21 with Σf(glycine) = 0 and Σ'f(arginine) = −1.10 respectively. Correlation coefficient, r = 0.9318.

*** Calculated by linear regression according to ref. 26, using $k'_{t.m} = ak'_{t.n} + b$, correlation coefficient, r = 0.9550.

discrepancies between the observed and predicted elution orders were apparent with other peptides, *e.g.* insulin (37) and insulin B chain (38) where the observed k' for 37 is less than k' for 38 which is opposite to that predicted on the basis of either set of summated indices. Retention anomalies of this type presumably reflect ionic peptide–stationary phase interactions such as those between protonated groups and free silanol groups and, with the larger peptides, the involvement of peptide secondary structures due to folding which modifies the number of exposed hydrophobic residues. Although the former effect can be minimised with adequate ligand surface carbon coverage, the generation of improved data banks of hydrophobicity parameters for peptides clearly would have to take conformational and related effects into account if selectivity predictions are to be any real value with larger polypeptides. Tables of these paramers will be reported elsewhere.

Organic solvent modifier

The retention behaviour of the various peptides and polypeptides (Table I) on octadecylsilica columns was determined using neat aqueous, aquo-methanol or aquo-acetonitrile mobile phases, at low pH containing 15–100 m*M* phosphate buffers, in isocratic, step- or gradient elution modes. In common with previous experiences[5,30], linear gradients gave better resolution with acetonitrile as the organic solvent modifier than exponential gradients. Dramatic changes in the k' values of some of the peptides, in accordance with the anticipated effect of γ on log k', were evident following relatively small changes in the acetonitrile or methanol concentrations. This was particularly noticeable with the more hydrophobic polypeptides (32–40) where efficient chromatography under isocratic conditions could only be carried out over a limited range of organic solvent concentrations if the k' values of particular peptides were to be kept within reasonable bounds, *e.g.* k' values below 10. Typical of the dependency of the k' of a peptide on the surface tension γ (and hence on the volume fraction, ψ) of

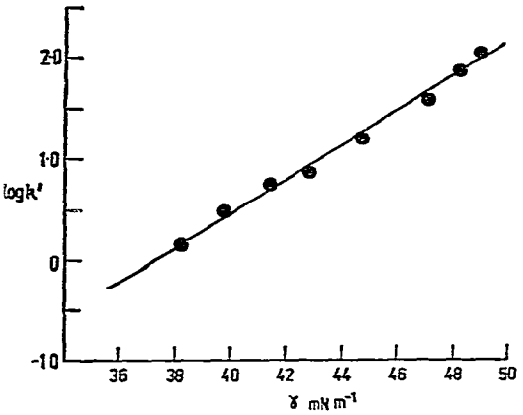

Fig. 1. Plot of log k' *versus* surface tension for peptide 28. Chromatographic conditions: column, μBondapak C_{18}; flow-rate, 1 ml/min; mobile phase, acetonitrile–water–50 m*M* NaH_2PO_4, with orthophosphoric acid to pH 2.5, run under isocratic conditions with 2% increases in acetonitrile content between each measurement. See Table I for peptide key.

acetonitrile– and methanol–water combinations is the plot of log k' *versus* γ for peptide 28 shown in Fig. 1. The chromatographic data for this peptide was obtained using isocratic conditions with step increase in the acetonitrile percentage composition and literature values[31] of the surface tensions. The good fit of the data, obtained with the above peptide, for a linear relationship between log k' and γ would suggest that the effect of the phosphate buffer, ionisation and other pH-dependent effects, are constant over the concentration range of acetonitrile examined. Over limited ranges of acetonitrile or methanol concentrations with low pH isocratic aquo–organic solvent mixtures, approximately linear dependencies of log k'_i on the volume fraction, ψ, of the organic modifier were also apparent for the various peptides used in this study. For peptides exhibiting relatively small k' values, *i.e.* those in the range $1 < k' < 10$, with neat aqueous mobile phase conditions, it should thus be possible to estimate the variation of log k' with ψ from the relationship[26]

$$\log k' = \log k'_0 - g\psi \tag{11}$$

(*cf.* eqns. 6, 10), although in the general case as a function of ψ, k' is probably more accurately expressed in terms of a quadratic dependency of log k' on ψ (ref. 30).

The chromatographic behaviour of the different peptides eluted under normal linear gradient conditions were also examined on the basis of anticipated quasi-linear relationships for $k'_{apparent}$ *versus* % organic solvent and log $k'_{apparent}$ *versus* γ. As can be seen from Figs. 2 and 3, the chromatographic data obtained for the series of small peptides (17–24) and polypeptides (25–40) are consistent with the basic premises. Furthermore, differences in the relative capacity factors of these peptides with acetonitrile and methanol linear gradients were in agreement with the observation[31] that the γ-values for acetonitrile–water mixtures are lower than for methanol–water

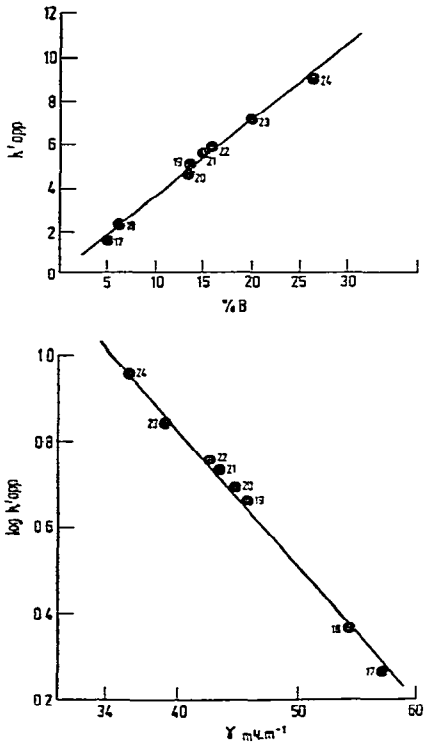

Fig. 2. Plots of k'_{app} *versus* percentage organic solvent modifier and log k'_{app} *versus* surface tension for the peptides 17–24. Chromatographic conditions: column, μBondapak C_{18}; flow-rate, 1 ml/min; mobile phase, water–50 m*M* NaH_2PO_4–0.1% H_3PO_4, to 50% acetonitrile–50% water–50 m*M* NaH_2PO_4–0.1% H_3PO_4 using a linear 60-min gradient. See Table I for peptide key.

mixtures at the same solvent percentage composition up to *ca.* 70% modifier but become higher above this percentage *i.e.* for 0–50% linear gradients the relative apparent k' values of the peptides were generally larger when methanol was used compared to acetonitrile. Solvent dependent selectivity changes were also noted with these two organic solvents. For example, the elution order for the peptides 17–24 was $17 < 18 < 19 < 20 < 21 < 22 < 23 < 24$ for acetonitrile gradient elution but $17 \approx 18 < 20 \approx 22 < 19 < 21 < 23 < 24$ for the corresponding methanol system. Similar selectivity changes have been observed with other hormonal peptides[8,32] and, in particular, with very hydrophobic polypeptides which require higher organic solvent concentrations to affect elution.

Effect of pairing ions on peptide retention

In previous studies[1,4,6] we have argued that the effect on peptide retention to alkyl-silicas of a number of anionic and cationic reagents, added to the mobile phase in low concentrations, *e.g.* between 0.05–5 m*M*, can be accommodated in terms of ion-pairing or dynamic liquid–liquid ion-exchange phenomena. Several of these reagents, *e.g.* hexane sulphonate, dodecylsulphate, dodecylammonium phosphate, are known[33,34] to act as surface active ions as revealed by Freundlich adsorption isotherms. Horváth

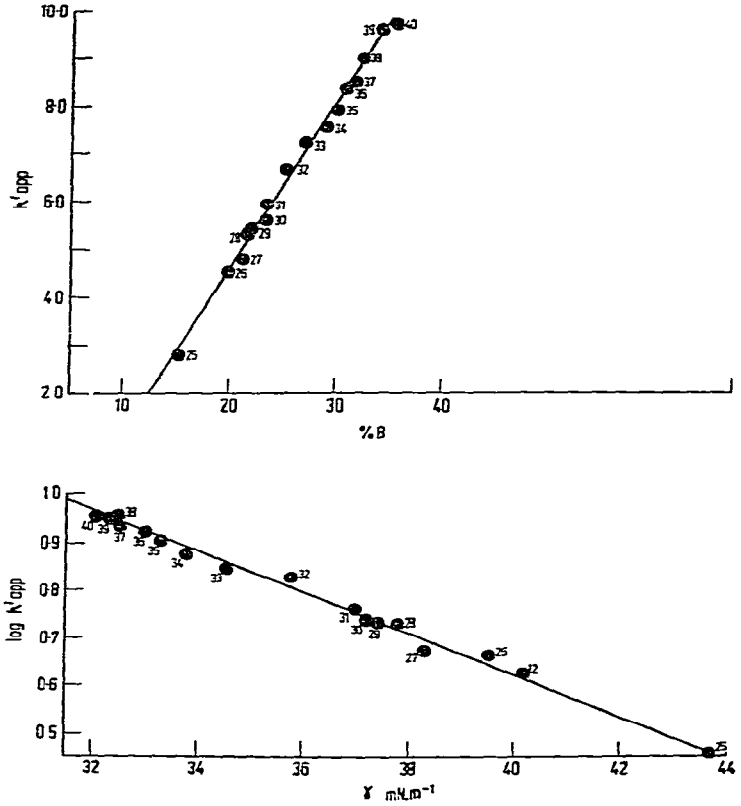

Fig. 3. Plots of k'_{app} *versus* percentage organic solvent modifier and log k'_{app} *versus* surface tension for the peptides 25–40. Chromatographic conditions as in Fig. 2.

et al.[35] have shown that the capacity factor for polar solutes such as catecholamines can be expressed as a function of the pairing ion concentration by

$$k' = (k_0 + \beta[X]) \cdot (1 + K_2[X])^{-1} \cdot (1 + K_3[X])^{-1} \quad (12)$$

where k_0 is the capacity factor in the absence of the counter-ionic species X, and β, K_2 and K_3 are equilibrium association constant terms describing the phenomenon. In circumstances where the adsorption of the pairing ion to the hydrocarbonaceous ligand is unfavoured, *i.e.* when $K_3[X] \ll 1$, then the retention behaviour can be described by

$$k' = (k_0 + \beta[X]) \cdot (1 + \gamma[X])^{-1} \quad (13)$$

Eqn. 12 is in the form of a parabolic function provided $(K_3[X])^{-1} \ll K_2^{-1}$ whilst eqn. 13 predicts a rectangular hyperbolic dependency of k' on [X]. For amino acids and simple dipeptides such as Pro-Tyr and Leu-Tyr, both hyperbolic and parabolic dependencies of k' on [X] have been observed[1,27,34]. As is apparent from Figs. 4 and 5, the retention characteristics of the peptides 1–24 on octadecylsilica columns in the

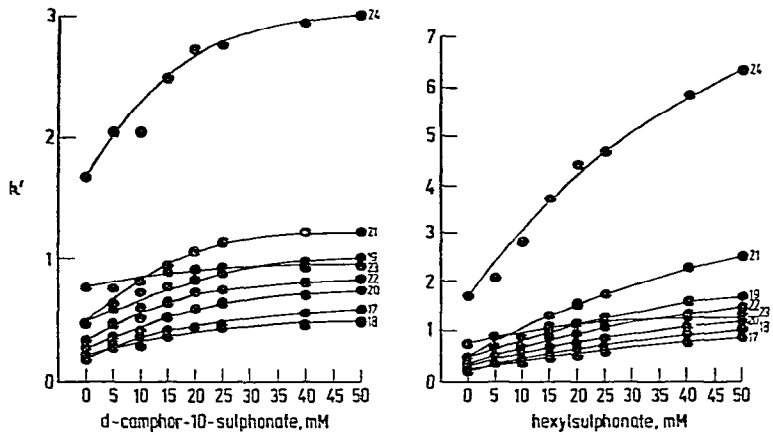

Fig. 4. Plots of the dependence of k' for peptides 17–24 on the concentration of D-camphor-10-sulphonate and hexylsulphonate. Chromatographic conditions: column μBondapak C_{18}; flow-rate, 2 ml/min; temperature 20°C; mobile phase, 50% methanol–50% water–50 m*M* NaH_2PO_4 with H_3PO_4 to pH 3.0 containing various concentrations of the ion-pairing reagents. See Table I for peptide key.

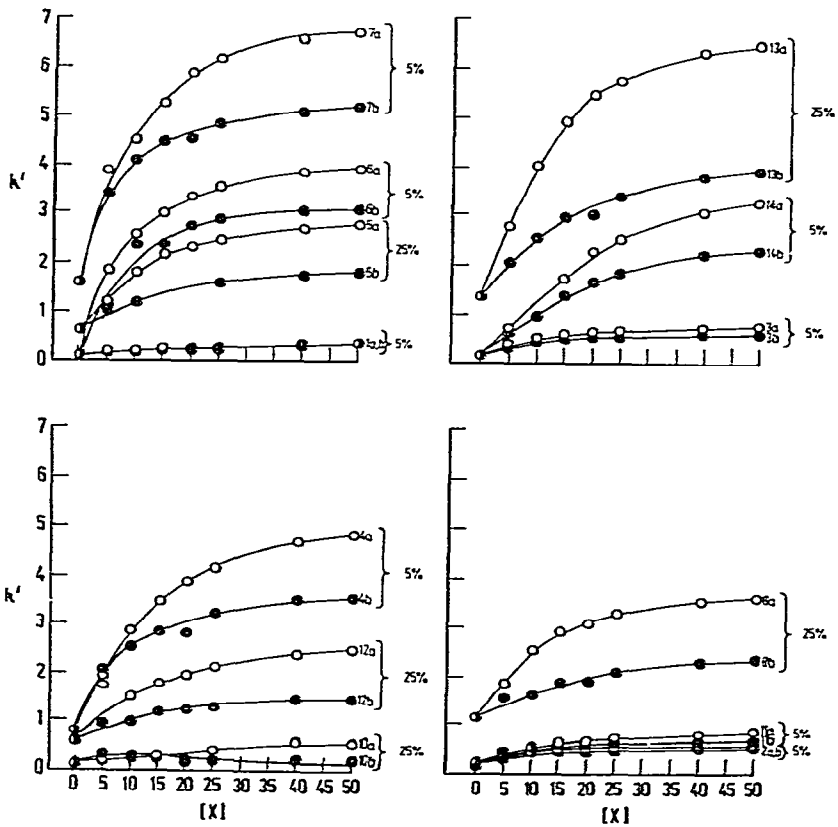

Fig. 5. Dependence of the capacity factors of protonated peptides on the concentration of ion-pairing reagents in the mobile phase. Chromatographic conditions similar to those in Fig. 4 with (a) corresponding to hexylsulphonate, (b) to D-camphor-10-sulphonate and the percentage methanol in the mobile phase shown in parenthesis. See Table I for peptide key.

presence of varying amounts of hexane sulphonate or D-camphor-10-sulphonate show essentially hyperbolic increases in k' up to pairing ion concentrations of 50 mM. Fig. 6 shows that the selectivity factors, log $\alpha_{i,j}$, (derived from $\log(k'_i/k'_j)$ where k'_i are the respective capacity factors for the peptides 18–24 at the various hexanesulphonate concentrations and k'_j is the capacity factor for peptide 17 at the same concentration) for these peptides are independent of the hexane sulphonate concentration above 20 mM. A similar trend is apparent with plots of log $\alpha_{i,j}$ *versus* [D-camphor-10-sulphonate]. This suggests that capacity factors can be altered independently of selectivity, at least for this series of peptides, by varying the pairing ion

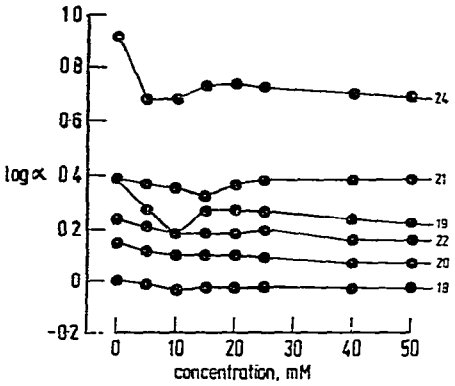

Fig. 6. Relationship between hexyl-sulphonate concentration and the logarithm of the selectivity factors for peptides 18–24 relative to peptide 17. Chromatographic conditions as in Fig. 4. See Table I for peptide key.

concentration. Similar effects have been noted[36] with weak organic acids and bases separated on reversed phases in the presence of pairing ions. Furthermore, it can be seen from Figs. 4 and 5 that the dependency of k' on [X] applies for peptides with free and protected C-termini in agreement with the concepts of anionic pairing ion interactions with protonated amino groups present in the peptide under the low pH conditions. An increase in the hydrophobicity of the pairing ion is known[36] to cause corresponding increases in the capacity factors of simple bases although solute selectivity is generally not affected. A similar situation is apparent with the peptides used in this study with in general k'_i (hexane sulphonate) $> k'_i$ (D-camphor-10-sulphonate) although some interesting selectivity changes were evident, *e.g.* $k'(22) > k'$ (23) with 50 mM hexanesulphate but the reverse is observed with *d*-camphor-10-sulphonate at the same concentration.

Effect of ionic strength

In contrast to the role of organic solvent modifiers, the effect of ionic strength on the retention characteristics of peptides on reversed phases has not yet been well delineated. By increasing the ionic strength, the surface tension of an aquo-organic solvent mobile phase is also increased, and with peptides advantage can be taken at high salt concentration of the "salting out" effect. Based on the relationship of log k'

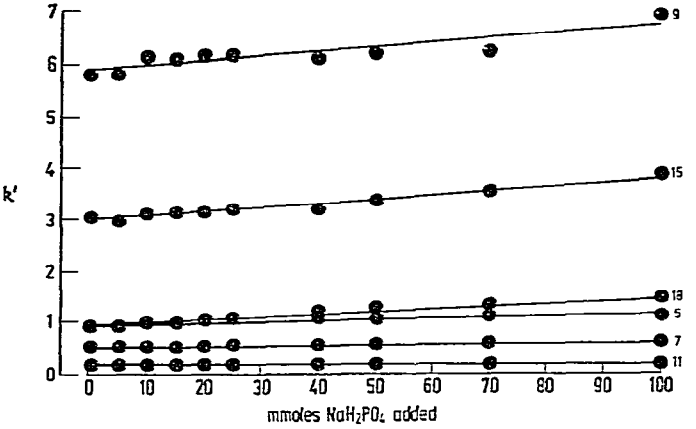

Fig. 7. Effect of ionic strength of the mobile phase on the retention of a group of peptides. Chromatographic conditions: column, μBondapak C_{18}; flow-rate, 2 ml/min; temperature, 20°C; mobile phase, 5% methanol–95% water–0.1% orthophosphoric acid with various concentrations of NaH_2PO_4 added. The pH at the initial phosphate concentration was pH 2.3 and at the final phosphate concentration pH 2.8. See Table I for peptide key.

versus γ, an increase in ionic strength should result in a rise of k' for a particular peptide. In the reversed-phase separation of the more hydrophobic polypeptides, *e.g.* 37 or 38, as well as in other studies[9,12] with smaller, non-polar peptides, the major salt effect that was noted for buffered mobile phases, was a slight decrease in k' values as the molarity of the buffer was increased. This effect does not appear to be invariant as is shown by the plots of k' *versus* $[NaH_2PO_4]$ for a series of simple peptides (Fig. 7). For many peptides listed in Table I, a general improvement in peak shape and column efficiency was observed. Most of the smaller peptides showed an insensitivity of their log $\alpha_{i,j}$ terms on ionic strength. However, some selectivity effects were apparent, *e.g.* the peptides 5 and 18 had coincident k' values at buffer concentrations up to 25 m*M* NaH_2PO_4 but were resolved when the concentration was progressively increased up to 100 m*M* NaH_2PO_4. Similarly, the larger polypeptides, *e.g.* 28–40 showed greater sensitivity of their log $\alpha_{i,j}$ terms on ionic strength. In practical terms, it is thus useful to choose several ionic strength conditions, and from an analysis of the k' data gauge regional responsiveness of the selectivity terms on ionic strength, so that a suitable buffer concentration can be selected for optimal resolution. With very complex peptide separations a compromise may be necessary with regard to different regions of the chromatogram. This point can be illustrated by comparison of the tryptic digest of human thyroglobulin run at 15 and 65 m*M* total phosphate concentration (Fig. 8a and b). Previously, we have described[1,4,5,37,38] a number of reversed-phase HPLC methods for the enzymatic profiling of proteins, including tryptic mapping, in which mobile phase conditions other than ionic strength were varied to control resolution. As can be seen in the above case, a simple change in ionic strength has caused a significant change in the elution profile. Although these two chromatograms are particularly complex in view of the large number of tryptic peptides formed from human thyroglobulin (which contains *ca.* 195 lysine residues and *ca.* 275 arginine residues per molecule[39]), these results do indicate that regional

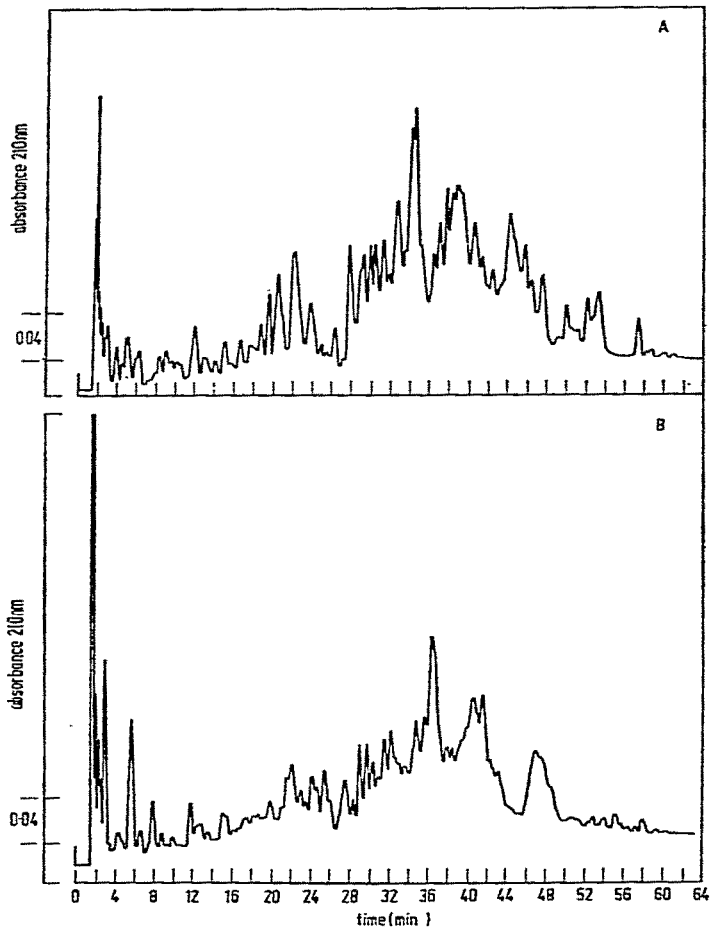

Fig. 8. Gradient elution profiles for the 200-min tryptic digest of human 19-S thyroglobulin. Column, μBondapak alkylphenyl; flow-rate, 2 ml/min; temperature, 20°C; elution conditions, a 60-min linear gradient was generated in (A) from water–0.1 % H_3PO_4 to 50 % acetonitrile–50 % water–0.1 % H_3PO_4 and in (B) NaH_2PO_4 (50 mmol/l) was added to the above mobile phase. Sample load, 300 μg in 60 μl.

selectivity changes can be induced by appropriate changes in the ionic strength. A discussion of the application of this effect in optimising the resolution of peptides of different composition or sequence, but of very similar hydrophobicities, will be deferred until later but it is worth pointing out that such ionic strength effects have been used successfully for the separation of recalcitrant tryptic peptides, from haemoglobin variants[37] and chick intestinal calcium binding proteins[40], by reversed-phase HPLC.

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