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EFFECTS OF ION-PAIRING REAGENTS ON THE PREDICTION OF PEPTIDE RETENTION IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have examined the resolution, on reversed-phase columns, of a series of model synthetic peptides and commercially available synthetic peptide standards under gradient elution conditions, using a water–acetonitrile mobile phase containing hydrophilic (phosphoric acid) or hydrophobic (trifluoroacetic acid, heptafluorobutyric acid) ion-pairing reagents. Increasing hydrophobicity or concentration of the ion-pairing reagents increased peptide retention times. It was clearly shown that these reagents effected changes in peptide retention time solely through interaction with the basic residues in the peptide. In general, each positive charge, whether originating from a lysine, arginine or histidine side-chain, or from an N-terminal α-amino group, exerts an equal effect on peptide retention. Different counterions have different effects on the change in peptide retention time per positively charged residue due to their differences in hydrophobicity. However, increasing concentrations of a specific counterion have an essentially equal effect per positively charged residue. These effects are also column dependent (*n*-alkyl chain length and ligand density).

These results, demonstrating a simple relationship between peptide retention in different ion-pairing systems, enabled the determination of rules for prediction of peptide retention times in one ion-pairing system from observed or predicted retention times in another system. The small average deviation of predicted and observed retention times for a series of basic peptides was good evidence for the value of this predictive method. This study provides a clear understanding of the effect of changing counterion hydrophobicity or concentration on peptide retention, and thus can be extremely beneficial in the purification of peptides and for providing proof of peptide homogeneity.

INTRODUCTION

The majority of researchers utilising ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC) at low pH for the separation of peptide mixtures take advantage of the excellent resolving power and selectivity of trifluoroacetic acid (TFA)–water to TFA–acetonitrile gradients at room temperature. Favoured models

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for the mechanism of ion-pair separations either involve formation of ion pairs with the sample solute in solution, followed by retention of the solute molecules on a reversed-phase column[1,2], or a dynamic ion-exchange event in which the ion-pairing reagent is first retained by the reversed-phase column and then solute molecules exchange ions with the counterion associated with the sorbed ion-pair reagent[3–6]. Both models yield similar predictions concerning separation as a function of experimental conditions. Whatever the mechanism, the resolving power of anionic ion-pairing reagents, including trifluoroacetate, is effected by its interaction with the basic residues of a peptide. In addition, the protonation of acidic residues increases the interaction of peptides with the reversed-phase support and the suppression of surface silanols at low pH (< 3.5–4.0) decreases ionic interactions with the support. If the presence of TFA is not sufficient to resolve efficiently a particular mixture of peptides, considerable flexibility in the degree of peptide retention and elution order may be achieved through careful choice of another anionic counterion.

We have previously reported[7,8] empirical sets of retention coefficients for amino acid residues at pH 2.0 and pH 7.0, obtained by measuring the effect of individual amino acid residues on the chromatographic behaviour of a series of model synthetic peptides in RP-HPLC. We have also demonstrated how appropriate modifications of our retention coefficients may be made to compensate for variations in chromatographic conditions which influence peptide resolution and retention (column length and diameter, *n*-alkyl chain length and ligand density, gradient steepness, flow-rate, temperature)[7,8]. The ability to predict the elution positions of peptides in the presence of different ion-pairing reagents would also be extremely useful. However, there are difficulties in predicting peptide retention values for a particular chromatographic system using coefficients derived from a different chromatographic system, particularly if the overall selectivities of the different systems diverge[9,10].

In this paper, we extend the utility of our retention coefficients, determined from a chromatographic system utilising TFA (0.1% v/v) as ion-pairing reagent[7], to the prediction of peptide retention in systems containing a more hydrophilic (orthophosphoric acid) or a more hydrophobic (heptafluorobutyric acid, HFBA) ion-pairing reagent. In addition, we examine the effect of counterion concentration on peptide resolution and retention.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). HPLC-grade TFA and Sequanal-grade HFBA were obtained from Pierce Chemical (Rockford, IL, U.S.A.). Reagent grade orthophosphoric acid was obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Peptides were prepared by solid-phase synthesis in this laboratory[7].

Synthetic peptide standards

A mixture of five synthetic peptide standards was obtained from the Alberta Peptide Institute (Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada). The composition of the peptides varied as follows: peptide S2, -Gly^3-Gly^4-; peptide S3, -Ala^3-Gly^4-; peptide S4, -Val^3-Gly^4-; peptide S5,

-Val[3]-Val[4]-. All peptides contained a N^{α}-acetylated N-terminal and a C-terminal amide, except peptide S1, which was identical to peptide S3 but had a free α-amino group. These standards are also available from Pierce (Rockford, IL, U.S.A.) and SynChrom (Linden, IN, U.S.A.).

Apparatus

The HPLC instrument consisted of a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) combined with a Varian 2080 column oven and coupled to a Hewlett-Packard (Avondale, PA, U.S.A.) HP 1040A detection system, HP85B computer, HP9121 disc drive, HP2225A Thinkjet printer and HP7470A plotter. Samples were injected with a 500-μl injection loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.).

Columns

Peptide mixtures were separated on two reversed-phase columns: (1) SynChropak RP-P C_{18}, 250 × 4.1 mm I.D., 6.5 μm particle size, 300 Å pore size, *ca.* 10% carbon loading (SynChrom, Linden, IN, U.S.A.); (2) Aquapore RP-300 C_8, 220 × 4.6 mm I.D., 10 μm, 300 Å (Brownlee Labs., Santa Clara, CA, U.S.A.).

RESULTS AND DISCUSSION

Effect of ion-pairing reagents on peptide retention

The labelling of a particular anionic counterion as hydrophobic or hydrophilic often tends to be somewhat arbitrary, relying as it does on the relative effectiveness of one ion-pairing reagent compared to another. TFA, for instance, has been described by various researchers as a hydrophilic, mildly hydrophobic or a hydrophobic ion-pairing reagent. All anionic counterions are potentially capable of ion-pairing with the positively charged basic residues of a peptide, thereby reducing its overall hydrophilicity and increasing peptide retention. However, they differ in their ability to interact with the reversed-phase support, thus producing a useful basis for defining the nature of a particular counterion. Hence, a hydrophobic counterion (*e.g.* trifluoroacetate, heptafluorobutyrate) is not only capable of ion-pairing with the basic solute, but, due to its hydrophobicity, can increase further the affinity of the peptides for the reversed-phase support. In contrast, a polar hydrophilic counterion (*e.g.* phosphate, chloride), following ion-pair formation with basic residues, would be unlikely to interact with the non-polar support. The increased peptide retention would only be due to reduction in hydrophilicity of positively charged residues by ion-pair formation. Previous studies on the use of perfluorinated carboxylic acids as ion-pairing reagents demonstrated increasing retention times of basic peptides with increasing hydrophobicity of the counterion[11–14]. HFBA has been used increasingly as the ion-pairing reagent of choice under circumstances where the resolving power of the TFA system has not been sufficient to separate satisfactorily a peptide mixture[13,15–19]. Apart from its effectiveness as an ion-pairing reagent, it shares with TFA the advantages of volatility and, at low concentrations, UV transparency to permit monitoring of column effluent at 210 nm. Despite being non-volatile, phosphoric acid has proved useful as a hydrophilic ion-pairing agent for hydrophobic peptides and proteins[9,10,13,20–24]. Its use, at 210 nm, permits a significant decrease in the concentration

of organic solvent in the mobile phase, thus reducing the possibility of denaturation or precipitation[21,22]. The effectiveness of orthophosphoric acid, TFA and HFBA as ion-pairing reagents were compared by examining their effect on retention of a series of model synthetic peptides, originally used to determine sets of retention coefficients in a 0.1% TFA system[7]: Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X is substituted by the 20 amino acids found in proteins. The peptides were eluted on a SynChropak C_{18} column (column 1; see Experimental) at a flow-rate of 1 ml/min and a temperature of 26°C, using a linear AB gradient (1% B/min), where A = 0.1% aq. orthophosphoric acid, TFA or HFBA and B = 0.1% of the three respective ion-pairing reagents in acetonitrile. The average contribution of each positive charge on the

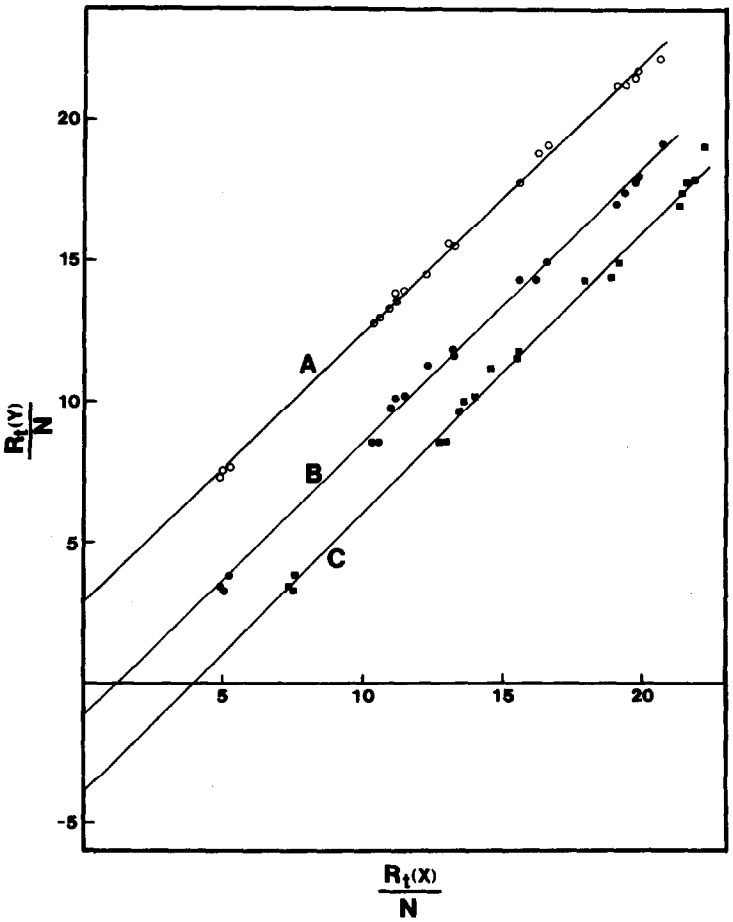

Fig. 1. Correlation of average contribution of each positively charged group to changes in peptide retention in RP-HPLC in the presence of different counterions. Conditions: column, SynChropak C_{18} (250 × 4.1 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% orthophosphoric acid, TFA or HFBA as ion-pairing reagent; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. R_t denotes retention time; N denotes number of positively charged groups in the peptide. Line A: y = HFBA; x = TFA; B: y = orthophosphoric acid, x = TFA, C: y = orthophosphoric acid, x = HFBA.

peptides to any change in their retention times in the presence of different counterions was determined by plotting R_t/N (R_t = peptide retention time; N = number of positive charges on the peptide) for each of the three ion-pairing reagents *versus* the values obtained for the other two reagents. The excellent degree of correlation (correlation coefficient = r) between the combinations of R_t/N for the three ion-pairing reagents is demonstrated in Fig. 1 (A compares HFBA and TFA, r = 0.999; B compares orthophosphoric acid and TFA, r = 0.998 and C compares orthophosphoric acid and HFBA, r = 0.997, calculated by linear least squares fitting). This high correlation, together with the similarity of the slopes (m) (A, m = 0.96, B, m = 0.98 and C, m = 1.01), suggests an essentially equal contribution by each positively charged residue to shifts in peptide retention when changing from one ion-pairing reagent to another. In addition, these results support the premise that, at low pH, only positively charged residues need be taken into account when determining the effect of various anionic counterions. The negligible change in retention time of a neutral peptide in the three-reagent systems further supported this view. The intercepts (b) of the plots on the ordinate (A, b = +2.83, B, b = −1.22 and C, b = −4.01) represent the average contribution of each anionic counterion per positively charged residue to changes in peptide retention. For example, to estimate the retention time of a peptide in an HFBA ion-pairing system (R_t^{HFBA}) from its observed retention time in a TFA system (R_t^{TFA}), the intercept of plot A (b = +2.83 min where y = HFBA, x = TFA) is multiplied by the number of positive charges (N) in the peptide and the value obtained added to R_t^{TFA}, *i.e.*, $R_t^{HFBA} = R_t^{TFA} + N\,(+2.83)$. Similar calculations may be made for other combinations of ion-pairing reagents, always keeping in mind that a change from a less hydrophobic to a more hydrophobic counterion will always have a positive effect on peptide retention time, while the reverse will reduce retention times, *e.g.* $R_t^{TFA} = R_t^{HFBA} + N\,(-2.83)$.

The concept of a simple correction factor to relate peptide retention times in different ion-pairing systems is at variance with studies on retention time prediction carried out by Browne *et al.*[17]. To account for the observed changes in the elution order of a series of 25 peptides, of varying sequence and length, in a water–acetonitrile gradient system containing either 0.1% TFA or 0.13% HFBA, these researchers applied linear regression analysis to estimate the contribution of each residue to peptide retention and presented different sets of retention coefficients for all the amino acid residues in both ion-pairing systems. The discrepancy between the results of Browne *et al.*[17] and those of the present study is due to the former researchers not identifying that the increased retention times of the peptides was solely a result of increased hydrophobicity of the peptides through ion-pair formation with the basic residues.

Requirement for peptide standards

Although the previous section demonstrated the viability of a simple relationship between peptide retention times in chromatographic systems containing different ion-pairing reagents, the correction factors for average contribution of each positive charge to changes in retention (intercepts of plots on ordinate; see Fig. 1) are only applicable to the particular column under investigation. Differences in peptide retention on different columns arise from a variety of factors, including column aging and variations in the ligand density or *n*-alkyl chain length of packing materials. It would

be impractical and time-consuming to chromatograph a large number of peptides whenever a new column is used, but the results demonstrated in Fig. 1 suggest that only a few standard peptides are needed to calculate the required retention time correction between different ion-pairing systems. A series of five synthetic peptide HPLC standards (S1–S5; see Experimental), designed for accurate monitoring of reversed-phase separations of peptides[25], were chromatographed on a new SynChropak C_{18} column (column 1) and an Aquapore C_8 column (column 2) under linear AB gradient conditions (A = 0.1% aq. orthophosphoric acid, TFA or HFBA and B = 0.1% of the respective ion-pairing reagents in acetonitrile; 1% B/min, 1 ml/min, 26°C). The elution profiles of the peptide standards on the two columns are shown in Fig. 2. Changes in the resolution and retention times of S1–S5 [taking into

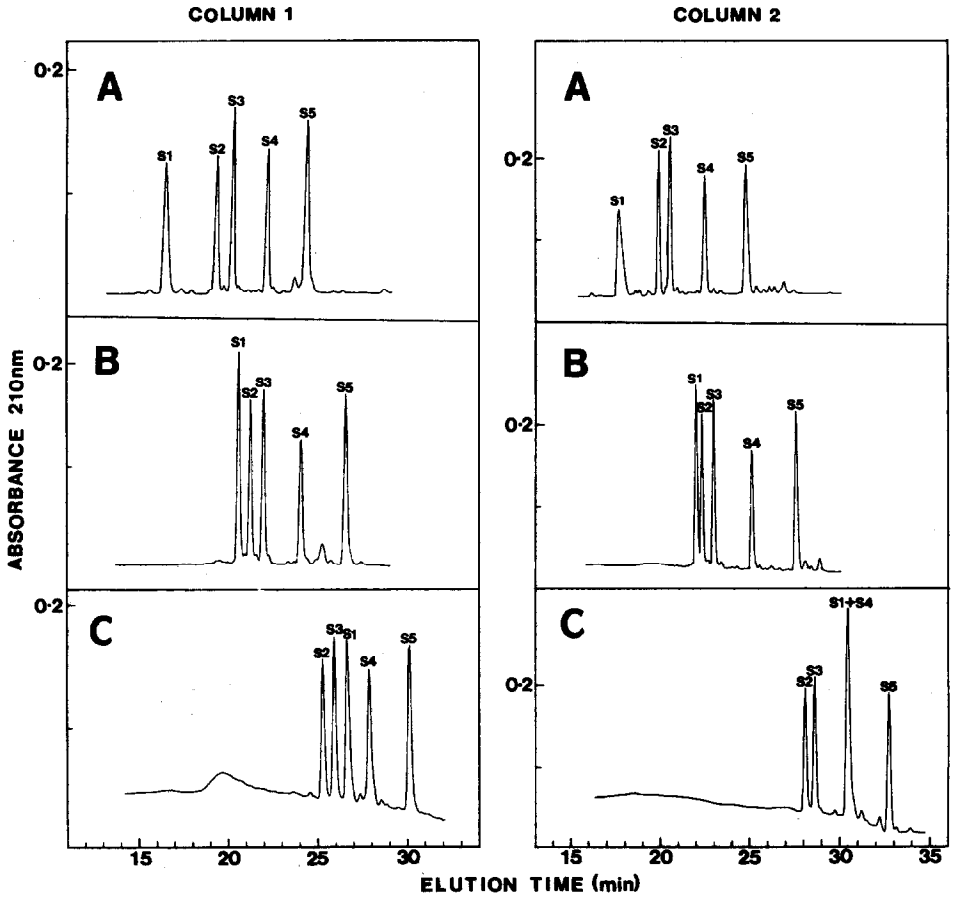

Fig. 2. Effect of ion-pairing reagents on the separation of a mixture of synthetic peptide HPLC standards in RP-HPLC. Conditions: column 1, SynChropak C_{18} (250 × 4.1 mm I.D.); column 2, Aquapore C_8 (220 × 4.6 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% orthophosphoric acid (panel A), TFA (B) or HFBA (C); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under Experimental.

TABLE I

EFFECT OF ION-PAIRING REAGENT ON RETENTION TIMES OF A SERIES OF SYNTHETIC PEPTIDE HPLC STANDARDS IN RP-HPLC

Conditions: column 1, SynChropak C_{18} (250 × 4.1 mm I.D.); column 2, Aquapore C_8 (220 × 4.6 mm I.D.); linear gradient, where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% orthophosphoric acid, TFA or HFBA as ion-pairing reagent; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under experimental.

Peptide standard	*Retention time (min)*			*Δ/N* (HFBA–TFA)*	*Δ/N (TFA–orthophosphoric acid)*
	Orthophosphoric acid	*TFA*	*HFBA*		
Column 1					
S1	16.3	20.5	26.7	2.1	1.4
S2	19.2	21.2	25.3	2.1	1.0
S3	20.0	21.9	25.9	2.0	1.0
S4	22.0	24.0	27.8	1.9	1.0
S5	24.2	26.5	30.1	1.8	1.2
				Average 2.0	Average 1.1
Column 2					
S1	17.8	21.9	30.4	2.8	1.4
S2	20.0	22.3	28.1	2.9	1.2
S3	20.7	22.9	28.6	2.9	1.1
S4	22.6	25.0	30.4	2.7	1.2
S5	24.8	27.5	32.7	2.6	1.4
				Average 2.8	Average 1.3

* Δ denotes difference in retention time of a peptide between two ion-pairing reagent systems; N denotes number of positively charged groups in peptide.

account, in particular, the position of the more highly charged S1 (three positively charged groups) in relation to the other four peptides (two positively charged groups)] supplies a rapid and convenient comparison of the effectiveness of anionic counterions. Although the elution orders of the peptides were identical on both columns, differences in overall resolution were apparent. The most dramatic difference in selectivity between the two columns was seen for the HFBA system (Fig. 2C), where S1 and S4 were either completely resolved (column 1) or ran as a single peak (column 2). The retention times of the five standards for all three ion-pairing systems are shown in Table I, together with the average contribution of each anionic counterion per positive charge to changes in peptide retention between the HFBA and TFA systems and between the TFA and orthophosphoric acid systems. These values were obtained by dividing the differences in retention time (Δ) of the peptide standards in two ion-pairing systems by the number of positive charges they possess (N), summating the resulting values ($\Delta^{S1}/N + \Delta^{S2}/N + \ldots. \Delta^{S5}/N$) and dividing this summated figure by 5. The final values obtained correspond to the plot intercepts (b) on the ordinate demonstrated in Fig. 1. The average contributions of each positively charged residue were, for the TFA ↔ HFBA systems, 2.0 min (column 1) and 2.8 min (column 2); for the TFA ↔ orthophosphoric acid systems, the values were 1.1 min (column 1) and 1.3 min (column 2). These values, applicable only to the re-

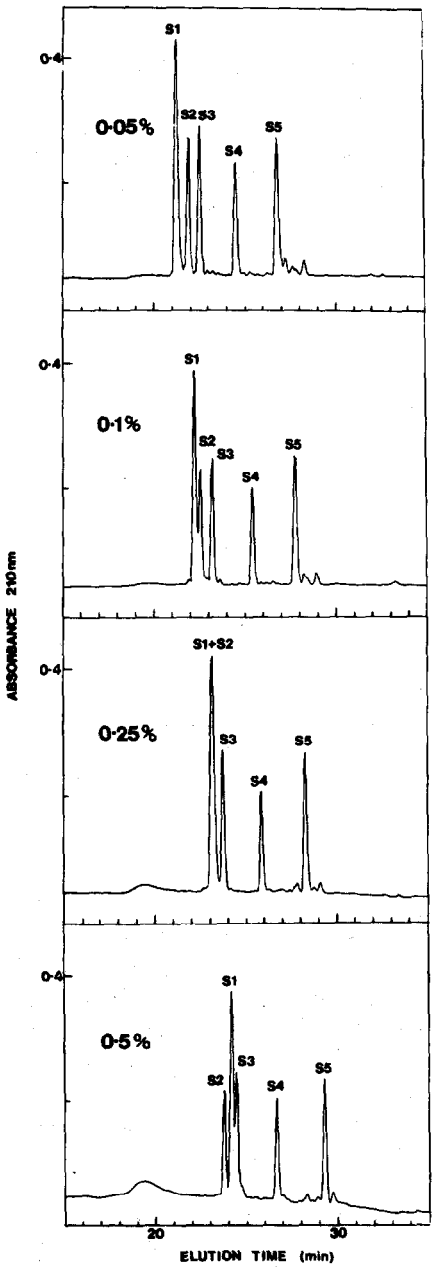

Fig. 3. Effect of concentration of ion-pairing reagent in RP-HPLC on the elution profile of a mixture of five synthetic peptide HPLC standards. Conditions: column, Aquapore C_8 (220 × 4.6 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.05%, 0.1%, 0.25% or 0.5% TFA; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequence variations of peptide standards S1–S5 are described under Experimental.

versed-phase columns from which they were determined, may be used to predict peptide retention times in one ion-pairing system from the observed or predicted retention times in another system (see later: Rules for Prediction of Peptide Retention Times).

Effect of concentration of ion-pairing reagent

Acidic ion-pairing reagents are generally used only at low concentrations (0.05–0.1% v/v) in the mobile phase. Although higher concentrations (1–2% v/v) are occasionally useful[24,26,27], lower levels of reagent help to prolong column life through decreased acidity of the mobile phase, without serious loss of column efficiency[28]. A number of researchers have demonstrated increasing peptide retention times with increasing concentrations of anionic counterions in the mobile phase[13,14,29,30]. Although these studies were applied to isocratic separations, the results are still pertinent to gradient elution systems and any dependence of peptide retention times on counterion concentration must be considered in retention time prediction. The effect of concentration of ion-pairing reagent on retention times of the five synthetic HPLC peptide standards (see Experimental) was examined on an Aquapore C_8 column (column 2; see Experimental). The peptides were eluted at a flow-rate of 1 ml/min and a temperature of 26°C with a linear AB gradient (1% B/min), where A = water and B = acetonitrile, both solvents containing 0.01–0.5% (v/v) of orthophosphoric acid, TFA or HFBA. Results for the TFA system are presented in Fig. 3. The peptides all demonstrated increasing retention times with increasing concentrations of the acids, with S1 (three positively charged groups) chang-

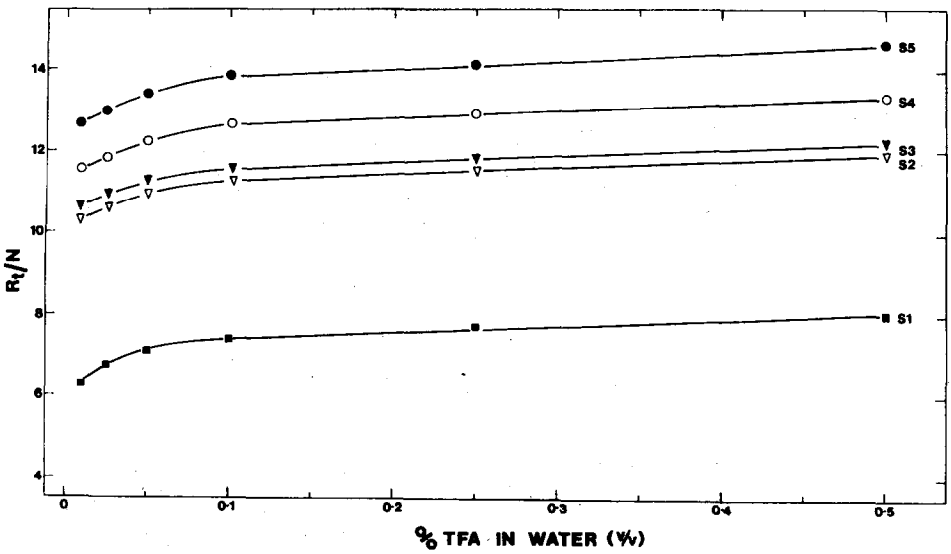

Fig. 4. A plot of the average effect per positively charged group of five peptide standards *versus* the concentration of ion-pairing reagent in RP-HPLC. Conditions: column, Aquapore C_8 (220 × 4.6 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.01–0.5% TFA; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. R_t denotes retention time; N denotes number of positively charged groups in peptide. Sequence variations of peptide standards S1–S5 are described under Experimental.

TABLE II

SEQUENCES OF PEPTIDES USED IN THIS STUDY

Ac = N^{α}-acetyl; amide = C^{α}-amide. Amino acid residues are denoted by the single letter code. Peptide 3 has a free α-NH_2 group.

Peptide	*Sequence*	*Number of positively charged groups*
0	Ac-T-D-L-L-G-amide	0
1	Ac-V-S-K-T-E-T-S-Q-V-A-P-A-amide	1
2A	Ac-R-G-A-G-G-L-G-L-G-K-amide	2
2B	Ac-R-G-V-G-G-L-G-L-G-K-amide	2
3	R-G-A-G-G-L-G-L-G-K-amide	3
4	Ac-S-D-Q-E-K-R-K-Q-I-S-V-R-G-L-amide	4
5	Ac-G-K-F-K-R-P-P-L-R-R-V-G-amide	5
6	Ac-G-K-F-K-R-P-P-L-R-R-V-R-amide	6

ing its position relative to the other four peptides (all possessing two positively charged groups) as the concentration increased. Dividing the retention times (R_t) of the peptides in the TFA system by the number of positively charged residues they possess (N), and plotting these values (R_t/N) *versus* concentration of TFA in the mobile phase produced the profiles shown in Fig. 4. The plots, similar for all three ion-pairing reagents, represent the average effect per positively charged residue of varying the counterion concentration. The similarity of the curves for all five peptide standards indicates an essentially equal effect of counterion concentration on each positively charged residue. These results not only demonstrate the value of the standards for monitoring counterion concentration effects, but also suggest that the resolution of some peptide mixtures may be improved simply by altering the counterion concentration in the mobile phase.

The potential resolving capability of variations in TFA concentration was examined by chromatographing several peptides of similar size but varying in the number of positively charged groups (Table II) on a new SynChropak C_{18} column (column 1; see Experimental). The peptides were eluted at a flow-rate of 1 ml/min and a temperature of 26°C using a linear AB gradient (1% B/min), where A = water and B = acetonitrile, both solvents containing 0.01–0.8% (v/v) of TFA. As demonstrated in Fig. 5, the peptides exhibited a changing resolution profile as the concentration of TFA in the mobile phase was increased, producing the best separation (baseline resolution of all five peptides) at a 0.05% concentration of the ion-pairing reagent. Plotting the retention times of the peptides *versus* TFA concentration in the mobile phase produced the overlapping profiles shown in Fig. 6A. Following an initial steep rise at low TFA concentrations (0.01–0.05%), the plots exhibited only a small change in peptide retention above levels of 0.2% of the ion-pairing reagent. This initial rise, starting, at 0.01% TFA, under conditions of possible counterion depletion[31] was greatest for peptide 6 (six positively charged residues) and decreased with decreasing basic character of the peptides. Plotting R_t/N *versus* TFA concentration (see Fig. 4) produced the profiles shown in Fig. 6B. The similarity of the profiles for the five peptides again suggests, for all practical purposes, an equal effect of counterion concentration on each basic residue. It may have been expected, due

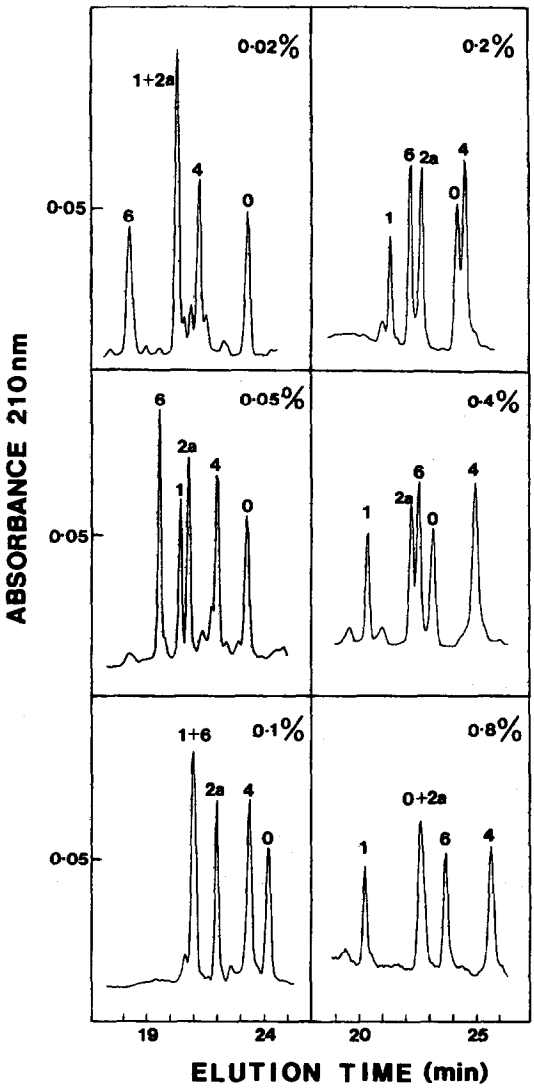

Fig. 5. Effect of concentration of ion-pairing reagent in RP-HPLC on the elution profile of a mixture of peptides with varying numbers of positively charged groups. Conditions: column, SynChropak C_{18} column (250 × 4.1 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.02, 0.05, 0.1, 0.2, 0.4 or 0.8% TFA; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequences of peptide are shown in Table II.

to the absence of any basic residues, that the retention times of peptide 0 would be unaffected by changes in TFA concentration. This peptide was certainly affected least over the TFA concentration range examined, but still exhibited a slight initial increase in retention time up to 0.2% TFA, followed by a slow decrease as the concentration increased to 0.8%. As the concentration of the ion-pairing reagent increased from 0.01–0.8% in the mobile phase, the pH of the aqueous component varied from 3.0

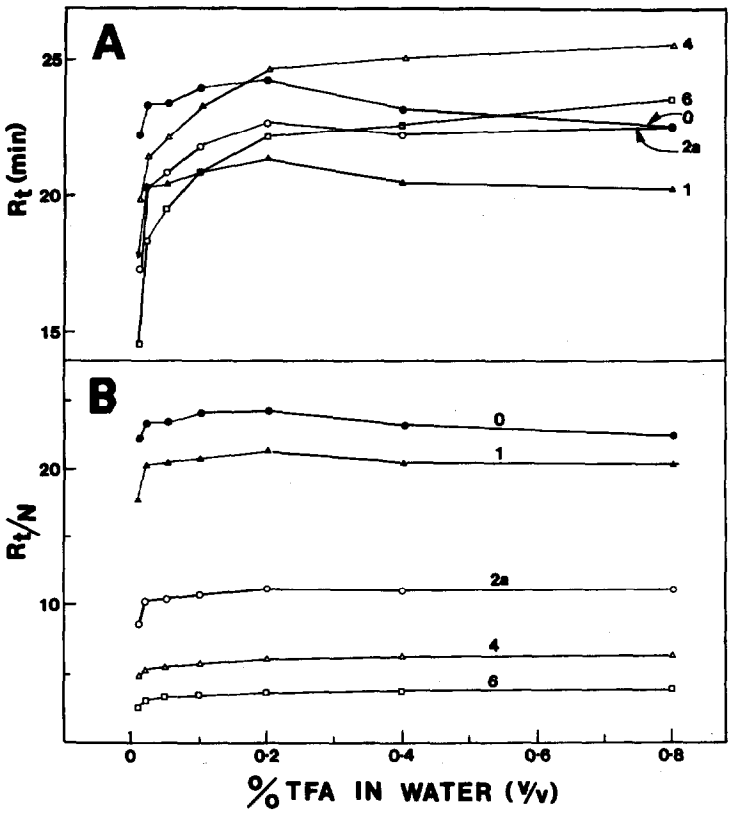

Fig. 6. Graphical presentation of the effect of concentration of ion-pairing reagent in RP-HPLC on retention times of five peptides with varying numbers of positively charged groups. (A) Retention time (R_t) *versus* concentration of TFA in mobile phase; (B) R_t/N *versus* concentration of TFA in mobile phase, where N denotes number of positively charged groups in peptide. Conditions: column, SynChropak C_{18} (250 × 4.1 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.01–0.8% TFA; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequences of peptides are shown in Table II.

to 1.3. Organic solvents tend to suppress ionization and the pH of an aqueous solvent mixture may only be an apparent value[11]. Although the apparent pH of the mobile phase may not be a very critical parameter provided that it is well below the pK of the peptide carboxyl groups (pK of Asp and Glu side-chain carboxyls varies between 4.0 and 4.4 for free amino acids and proteins, respectively; for C-terminal α-carboxyl groups, the pK varies between 2.0 and 3.1, respectively) it is possible that the combination of increasing concentration of the hydrophobic trifluoroacetate counterion, coupled with a significant drop in pH, is somehow affecting the interaction of the reversed-phase support with the peptide. These results demonstrate clearly the importance of consistency in the concentration of ion-pairing reagent in the mobile phase for accurate run-to-run comparisons of peptide separations.

Prediction of peptide retention times

The degree of shift in peptide retention time with changes in counterion is dependent on counterion hydrophobicity, counterion concentration and the selectivity of the particular reversed-phase column used. The following rules for prediction of peptide retention in one counterion system from the results of another system require two basic assumptions: first, only basic, positively charged residues contribute to shifts in peptide retention; secondly, each positive charge, whether originating from a lysine, arginine or histidine side-chain, or from an N-terminal α-amino group, exerts an equal effect on peptide retention. The retention coefficients reported by Guo *et al.*[7] were determined using 0.1% TFA (v/v) as ion-pairing reagent in the water–acetonitrile mobile phase. To avoid any complications arising from counterion concentration effects, it is recommended that this level of ion-pairing reagent be consistently used to simplify peptide retention time prediction.

The retention time (τ) for a peptide in the counterion system used to determine a particular set of coefficients equals the sum of the retention coefficients (ΣR_c) for the amino acid residues and end groups[7] plus the time for elution of unretained compounds (t_0) and the time correction for the peptide standard (t_s),

$$\tau = \Sigma R_c + t_0 + t_s$$

The contribution of each positively charged residue to shifts in peptide retention is determined by chromatographing a basic peptide standard with both the counterion of choice and the counterion used to determine the retention coefficients. The average contribution of each basic residue to a change in retention time is denoted by Δ/N, where Δ is the shift (in min) in retention time of the standard between the two counterion systems, and N equals the number of positively charged residues in the standard. The counterion correction factor (t_i) for a peptide of interest is then obtained by multiplying the number of positively charged residues of the peptide (n) by Δ/N for the standard,

$$t_i = n(\Delta/N).$$

Thus, the predicted retention time of a peptide in the second counterion system is described by the expression,

$$\tau = \Sigma R_c + t_0 + t_s + t_i$$

Rule for prediction when varying counterion

This rule applies to linear AB gradients at 1% B/min from a starting composition of 100% A (where A and B are water and acetonitrile, respectively, each containing the ion-pairing reagent), a flow-rate of 1 ml/min, and a temperature of 26°C.

When the retention time of a peptide of interest is known in the presence of one counterion, its predicted position in another counterion system is described by the expression,

$$\tau = R_t^{obs} + t_i,$$

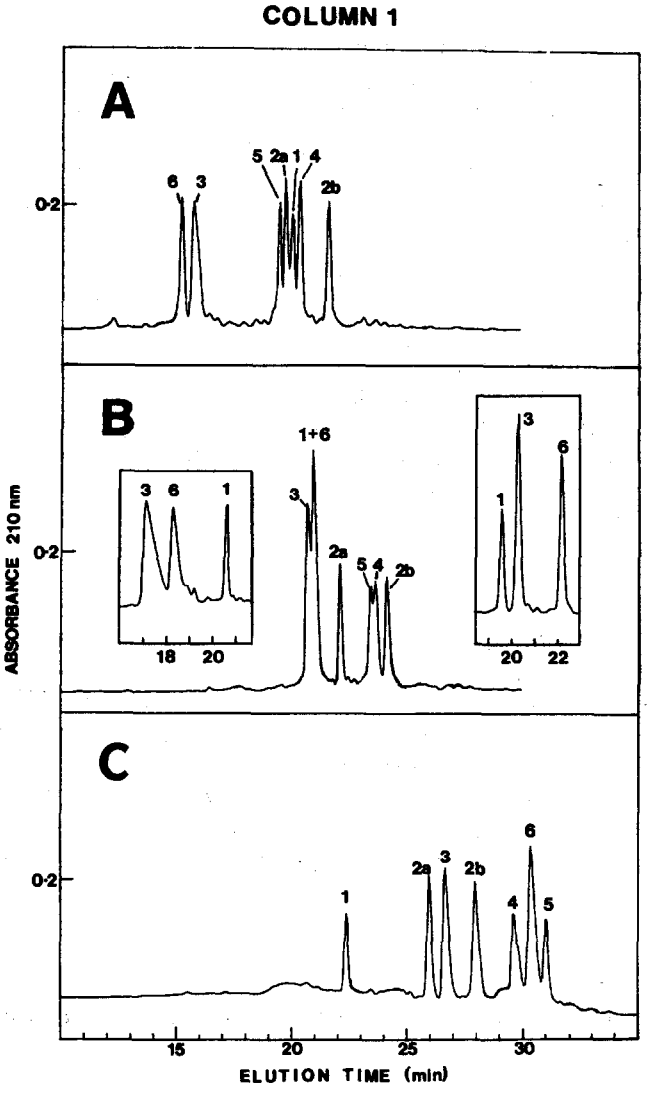

Fig. 7. Effect of ion-pairing reagent on the separation of a mixture of basic peptides in RP-HPLC. Conditions: column, SynChropak C_{18} (250 × 4.1 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% orthophosphoric acid (Panel A), TFA (B) or HFBA (C); flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Panel B, insets: left, 0.01% TFA in solvents A and B; right, 0.4% TFA in solvents A and B. Sequences of peptides are shown in Table II.

where τ is the predicted peptide retention time in the desired counterion, R_i^{obs} is the observed retention time in another counterion system, and t_i is the counterion correction factor (see above).

This procedure is preferable to that outlined before (Prediction of Peptide Retention Times) since any deviation between the predicted and observed retention times of peptides in the counterion system used to determine the retention coefficients

would be further amplified when converted to predicted values in another system. In addition, slight variations in counterion concentration would also introduce further errors in the accuracy of the method.

Accuracy of peptide retention prediction

To examine the accuracy of retention time prediction between systems containing different ion-pairing reagents, the above prediction rules were applied to the separation of a mixture of basic peptides of varying numbers of positively charged groups (Table II) on a new SynChropak C_{18} column and an Aquapore C_8 column (columns 1 and 2, respectively; see Experimental). The peptides were eluted at a flow-rate of 1 ml/min and a temperature of 26°C using a linear AB gradient where A and B are water and acetonitrile, respectively, containing 0.1% (v/v) of orthophosphoric acid, TFA or HFBA. The results for column 1 are shown in Fig. 7 and demonstrate increasing retention times of the peptides with increasing hydrophobicity of the counterion: $HFBA^-$ (Fig. 7C) > TFA^- (Fig. 7B) > $H_2PO_4^-$ (Fig. 7A). In addition, the elution order of the peptides changed from one counterion system to another. This is clearly illustrated in Fig. 7, where the elution order of peptides 1, 3 and 6 (containing one, three and six positively charged residues, respectively) was reversed as the counterion changed from $H_2PO_4^-$ (Fig. 7A) to $HFBA^-$ (Fig. 7C). In addition, the elution order is changed by changing the counterion concentration. The

TABLE III

EFFECT OF ION-PAIRING REAGENT ON PREDICTED AND OBSERVED RETENTION TIMES OF BASIC PEPTIDES IN RP-HPLC

Conditions: column 1, SynChropak C_{18} (250 × 4.1 mm I.D.); column 2, Aquapore C_8 (220 × 4.6 mm I.D.); linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing 0.1% orthophosphoric acid, TFA or HFBA as ion-pairing reagent; flow-rate, 1 ml/min; 26°C; absorbance at 210 nm. Sequences of peptides are shown in Table II.

Peptide	R_t^{obs}* *TFA*	τ** *Orthophosphoric acid*	R_t^{obs} *Orthophosphoric acid*	τ *HFBA*	R_t^{obs} *HFBA*
Column 1					
1	21.0	19.9	20.1	23.0	22.4
2a	22.1	19.9	19.8	26.1	26.0
2b	24.1	21.9	21.7	28.1	28.0
3	20.8	17.5	15.8	26.8	26.7
4	23.6	19.2	20.4	31.6	29.6
5	23.5	18.0	19.5	33.5	30.4
6	21.0	14.4	15.3	33.0	31.0
Column 2					
1	20.5	19.2	19.8	23.3	23.7
2a	22.5	19.9	19.3	28.1	28.9
2b	25.6	23.0	21.9	31.2	30.4
3	21.3	17.4	15.6	29.7	29.9
4	24.0	18.8	19.3	35.2	32.2
5	23.7	17.2	17.6	37.7	35.6
6	20.5	12.7	12.6	37.3	35.6

* R_t^{obs} denotes observed retention time of a peptide.

** τ denotes predicted retention times of peptides, calculated as described in text.

elution order in the right inset of Fig. 7B was 1, 3 and 6 at 0.4% TFA, compared to 3, 6 and 1 at 0.01% TFA in the left inset of Fig. 7B. In many cases in the purification of synthetic peptides, contaminating peptides and the desired peptide can be very similar in hydrophobicity under the conditions used. If the contaminants vary in the number of positively charged residues they contain compared to the peptide of interest, changing the counterion hydrophobicity or concentration should resolve these contaminants from the desired peptide. This approach is probably more advantageous than searching for columns with different selectivities and, in addition, is a useful test of peptide homogeneity. The predicted retention times (τ) of the peptides in the orthophosphoric acid and HFBA systems were calculated and compared to the observed retention values (R_t^{obs}) (Table III). The values for Δ/N (retention time shift/basic residue) were obtained from Table I and represent the average value of Δ/N for all five synthetic peptide HPLC standards. This approach should ensure greater accuracy than the use of just one standard. The results presented in Table III illustrate the accuracy of the method for the peptides examined. The average deviations of predicted and observed values for the seven peptides on column 1 were 0.8 min (TFA → orthophosphoric acid) and 1.1 min (TFA → HFBA); for column 2, the values were 0.7 min (TFA → orthophosphoric acid) and 1.3 min (TFA → HFBA). The results for the prediction method showed generally good accuracy and would certainly be extremely useful in narrowing down the position of a peptide(s) of interest in the elution profile of a peptide mixture. The large change in retention time of highly basic peptides, observed on changing the counterion, is shown in Table III. For example, the retention times of peptide 6 (six positively charged groups) are 12.7, 20.5 and 35.6 min, respectively, for the ion-pairing reagents orthophosphoric acid, TFA and HFBA. By comparison, the retention times of peptide 1 (one positively charged group) are 19.8, 20.5 and 23.7, respectively.

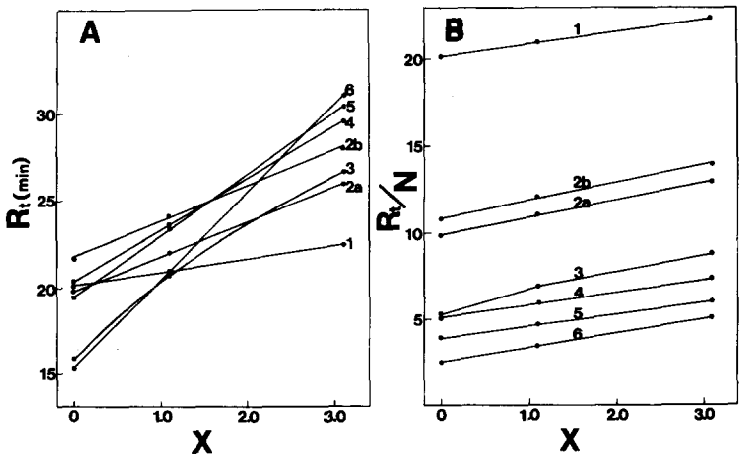

Fig. 8. Graphical presentation of the effect of hydrophobicity of ion-pairing reagent on peptide retention in RP-HPLC. (A) Retention time (R_t) *versus* the average increase in retention time (X) per positively charged group, obtained from the peptide standards S1–S5, when TFA or HFBA was used as the mobile phase acid compared to the retention times of the peptide standards when orthophosphoric acid was used as the mobile phase acid; X = 1.1 (TFA) and 3.1 (HFBA) (Table I). (B) R_t/N *versus* X, where N denotes number of positively charged groups in peptide. Sequences of peptides are shown in Table II.

The seven peptides used in this study varied in both the numbers and types of basic residues they contained. Discrepancies between predicted and observed peptide retention times may be due to slightly unequal contributions of these residues. The observed retention times (R_t^{obs}) of the seven peptides in orthophosphoric acid, TFA and HFBA (Table III) were plotted against the average increase in retention time per positively charged residue (X) (obtained from peptide standards S1–S5, with TFA or HFBA as counterion, see Table I) compared to the retention times of the peptide standards when orthophosphoric acid is used as the mobile phase acid (for TFA, X = 1.1; for HFBA, X = 1.1 + 2.0 = 3.1, see Table I). As shown in Fig. 8A, the slopes of the plots for each peptide are very different, depending as they do on the number of positive charges present in the peptides. With the exception of peptide 3, the plots illustrate a linear relationship between peptide retention time and counterion hydrophobicity. If X is now plotted against R_t/N (where N is number of positively charged residues in the peptides), the profiles shown in Fig. 8B are obtained. The similar, if not absolutely parallel, slopes for most of the peptides justifies, for most practical purposes, the assumptions required to simplify peptide retention prediction, *i.e.* only basic residues need be taken into account and each residue exerts an essentially equal effect on retention. It is possible that the very close proximity of two charged groups (an α-amino group on an N-terminal Arg residue) is producing the anomalous results exhibited by peptide 3. It has already been demonstrated[7] that a charged α-amino group on a basic N-terminal residue has a different effect on peptide retention at pH 2 and pH 7 than an α-amino group on an N-terminal residue with an uncharged side chain. However, the small average deviation of predicted and observed peptide retention times demonstrated in Table III is good evidence for the usefulness of this predictive method, and provides for the first time a clear understanding of the major effect of changes in counterion and counterion concentration on the retention of peptides in RP-HPLC.

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