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Journal of Chromatography A, 1080 (2005) 68–75
JOURNAL OF CHROMATOGRAPHY A
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Effect of anionic ion-pairing reagent hydrophobicity on selectivity of peptide separations by reversed-phase liquid chromatography

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Available online 20 April 2005

Abstract

Despite the continuing dominance of trifluoroacetic acid (TFA) as the anionic ion-pairing reagent of choice for peptide separations by reversed-phase high-performance liquid chromatography (RP-HPLC), we believe that a step-by-step approach to re-examining the relative efficacy of TFA compared to other ion-pairing reagents is worthwhile, particularly for the design of separation protocols for complex peptide mixtures, e.g., in proteomics applications. Thus, we applied RP-HPLC in the presence of different concentrations of anionic ion-pairing reagents – phosphoric acid, TFA, pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) – to a mixture of three groups of four 10-residue peptides, these groups containing peptides of +1, +3 or +5 net charge. Overall separation of the 12-peptide mixture improved with increasing reagent hydrophobicity (phosphate$^-$ < TFA$^-$ < PFPA$^-$ < HFBA$^-$) and/or concentration of the anion, with reagent hydrophobicity having a considerably more pronounced effect than reagent concentration. HFBA, in particular, achieved an excellent separation at a concentration of just 10 mM, whereby the peptides were separated by charged groups (+1 < +3 < +5) and hydrophobicity within these groups. There was an essentially equal effect of reagent hydrophobicity and concentration on each positive charge of the peptides, a useful observation for prediction of the effect of varying counterion concentration hydrophobicity and/or concentration during optimization of peptide purification protocols. Peak widths were greater for the more highly charged peptides, although these could be decreased significantly by raising the acid concentration; concomitantly, peptide resolution increased with increasing concentration of ion-pairing reagent.

Keywords: Reversed-phase liquid chromatography; Peptides; Anionic ion-pairing reagents; TFA; PFPA; HFBA

1. Introduction

Our laboratory is currently taking advantage of the commercial availability of silica-based reversed-phase packings developed for excellent chemical stability at acidic pH values [1–3] to revisit the question of the most suitable type and concentration of acidic anionic ion-pairing reagent for separation of peptide mixtures. Despite the dominance of trifluoroacetic acid (TFA) as the anionic ion-pairing regent of choice for peptide separations [4–6], we believe that a step-by-step approach to re-examining the relative efficacy of TFA compared to other ion-pairing reagents is worthwhile. The importance of such an undertaking cannot be underestimated, particularly considering the range of positively charged peptides which may be present in peptide mixtures – in proteomic applications, for instance, where protein digests may contain thousands of peptides with multiple charges.

The perfluorinated homologous series of acids represents a useful series of anionic ion-pairing reagents used for peptide separations, with TFA the most commonly employed, but pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) have also seen occasional use [4,6–12]. The negatively charged trifluoroacetate (TFA$^-$), pentafluoropropionate (PFPA$^-$) or heptafluorobutyrate (HFBA$^-$) anion will interact (ion-pair) with positively charged peptide residues (arising from the basic side-chains Lys, Arg and His, or a free α-amino group). Such hydrophobic anions will not only neutralize the positively charged groups, thereby decreasing peptide hydrophilicity, but will increase further the affinity of the peptides for the reversed-phase sorbent [10]. In addition, more hydrophilic anionic ion-pairing reagents such

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doi:10.1016/j.chroma.2005.03.035

as phosphoric acid (producing the negatively charged phosphate anion) have also seen use for specific peptide applications in RP-HPLC [4–6,10,13–18], permitting a significant decrease in the concentration of organic solvent in the mobile phase, thus reducing the possibility of denaturation or precipitation [13]. Thus, the hydrophobicity of the anions employed in the present study increased in the order of phosphate < TFA^- < $PFPA^-$ < $HFBA^-$, offering a useful range of anion hydrophobicity in our efforts to delineate the effects of ion-pairing reagent hydrophobicity and concentration on selectivity of peptide separations.

In the present study, we applied RP-HPLC in the presence of different concentrations of phosphoric acid, TFA, PFPA and HFBA to a mixture of three groups of model peptides, these groups containing peptides of +1, +3 or +5 net charge. From the retention behaviour of these peptides, conclusions could be drawn about optimum approaches to the separation of sample mixtures containing peptides of varying net charge and hydrophobicity.

2. Experimental

2.1. Materials

Reagent-grade phosphoric acid (H_3PO_4) was obtained from Caledon Laboratories (Georgetown, Ont., Canada). TFA was obtained from Hydrocarbon Products (River Edge, NJ, USA); PFPA was obtained from Fluka (Buchs, Switzerland); and HFBA was obtained from Pierce Chemical (Rockford, IL, USA). HPLC-grade water was obtained from EMD Chemical (Gibbstown, NJ, USA). HPLC-grade acetonitrile was obtained from EM Science (Gibbstown, NJ, USA).

2.2. Column and HPLC conditions

Analytical RP-HPLC runs were carried out on a Zorbax SB300-C_8 column (150 mm × 2.1 mm I.D.; 5 μm particle size, 300 Å pore size) from Agilent Technologies (Little Falls, DE, USA), using a linear AB gradient (0.5% acetonitrile/min) at a flow-rate of 0.3 ml/min, where Eluent A was 10, 20 or 30 mM aq. H_3PO_4, TFA, PFPA or HFBA and Eluent B was the corresponding concentration of the respective ion-pairing reagent in acetonitrile; runs were carried out at 25 °C. Approximately 1 μmol of each of the peptides in the peptide mixture was injected in a total sample value of 10 μl. The results presented were obtained from at least duplicate HPLC runs.

2.3. Instrumentation

RP-HPLC runs were carried out on an Agilent 1100 Series liquid chromatograph. Peptide synthesis was carried out on an Applied Biosystems Peptide synthesizer Model 430A (Foster City, CA, USA).

2.4. Peptide synthesis and purification

Peptide synthesis was carried out by standard solid-phase synthesis methodology using N^{α}-*tert*-butyloxycarbonyl (*t*-Boc) chemistry on MBHA (methylbenzhydrylamine) resin (0.97 mmol/g) as described previously [19]. The crude peptides were purified by preparative RP-HPLC on an Applied Biosystems 400 solvent-delivery system connected to a 783A programmable absorbance detector. Amino acid analyses of purified peptides were carried out on a Beckman Model 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA, USA) and the correct primary ion molecular masses of peptides were confirmed by mass spectrometry on a Mariner Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA).

2.5. Calculation of resolution (R_s)

Resolution was calculated through the equation:

$$R_s = \frac{1.176\,\Delta t_R}{W_1 + W_2}$$

where Δt_R is the difference in retention time between two peptide peaks (1 and 2) and W_1 and W_2 are their peak widths at half height [4]. This equation is satisfied if the units of retention time and peak width are the same, such as minutes. The peak widths at half height (in time units) used in this study were determined using the HP ChemStation for LC Systems software package Rev. 0701.

3. Results and discussion

3.1. Design of synthetic model peptides

We believe that studies designed to correlate peptide elution behaviour in RP-HPLC with varying run parameters is best achieved by studies using defined model peptide systems. The results from such model peptides can then be extrapolated to peptides as a whole. Thus, the three groups of model peptides in Table 1 exhibit variations in hydrophobicity and net positive charge. From Table 1, each group of peptides contains four peptides with the same net positive charge, arising from the presence of a single lysine residue (+1 group), two lysine residues and an arginine residue (+3 group) or two lysine residues, two arginine residues and a free N-terminal α-amino group (+5 group). Within each peptide group, hydrophobicity varies only subtly between adjacent peptides, particularly within the +1 and +3 peptide groups where peptide hydrophobicity varies by just one methyl or methylene group (equivalent to an increase of one carbon atom) from one peptide to the next. The presence of several glycine residues ensures negligible secondary structure for these peptides [20,21], i.e., they have a "random coil" configuration, to avoid potential complications in data interpretation due to selectivity differences in peptide RP-HPLC

Table 1
Sequences and names of the peptides in this study

Peptide group[a]	Peptide name	Peptide sequence[b]
+1	1a	Ac-GG**GG**GLGLGK-amide
	1b	Ac-GG**AG**GLGLGK-amide
	1c	Ac-GG**AA**GLGLGK-amide
	1d	Ac-GG**VG**GLGLGK-amide
+3	3a	Ac-GR**GG**KLGLGK-amide
	3b	Ac-GR**AG**KLGLGK-amide
	3c	Ac-GR**AA**KLGLGK-amide
	3d	Ac-GR**VG**KLGLGK-amide
+5	5a	NH_3^+-RR**GG**KLGLGK-amide
	5e	NH_3^+-RR**VA**KLGLGK-amide
	5h	NH_3^+-RR**VV**KLGLGK-amide
	5j	NH_3^+-RR**II**KLGLGK-amide

[a] The charge of the peptide is shown at pH 2.0.
[b] The different amino acid substitutions are shown in bold letters.

retention behaviour arising from conformational variations [18,22]. The 10-residue length of the peptides was chosen to mimic the size of an average peptide fragment arising from proteolytic digests of proteins. Peptides are denoted by charge and relative hydrophobicity order, e.g., the peptide with one positive charge and the lowest hydrophobicity within this +1 group (a –GG-substitution; Table 1) is denoted 1a; the peptide with three positive charges and the highest hydrophobicity within this +3 group (a –VG-substitution; Table 1) is denoted 3d, etc. Within the +5 peptide group (arising from two lysine residues, two arginine residues and a free α-amino group at the N-terminal), peptide hydrophobicity increases in the order 5a (a –GG-substitution) < 5e (VA) < 5h (VV) < 5j (II).

3.2. RP-HPLC stationary phase

The Zorbax SB-300 C_8 ("SB" denoting "Stable Bond") is prepared from monofunctional n-octylsilane based on protecting the siloxane bond between the silica and the C_8 group with bulky side groups, in this case two isopropyl groups [1–3]. This packing was originally designed to protect the siloxane bond at low pH [1–3], thus overcoming concerns of stationary phase degradation through cleavage of alkyl chains from silica-based packings via acid hydrolysis of this bond linking the stationary phase functional group with surface silanols [23,24]. In our hands, this packing has shown excellent stability when employing acidic mobile phases containing up to 0.25% TFA [12] and was therefore the packing of choice for the present study.

3.3. Effect of hydrophobicity of ion-pairing reagent on peptide elution behaviour

The effect of increasing hydrophobicity of the ion-pairing reagent on the elution behaviour of the 12-peptide mixture is shown in Fig. 1. We chose to express the concentration of the ion-pairing reagents in mM (10 mM in the case of Fig. 1) instead of the traditional % value in order to be able to make a direct comparison of the effectiveness of the four reagents.

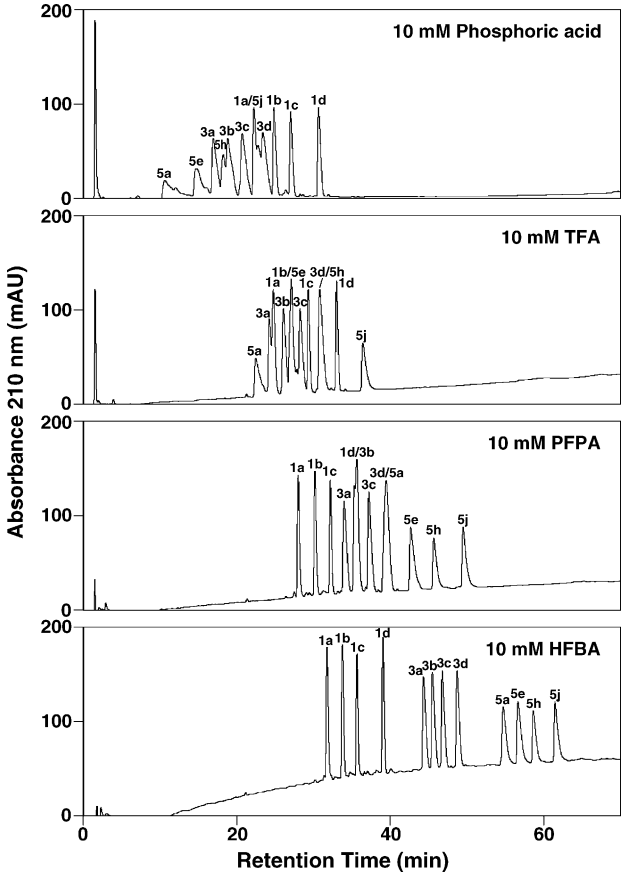

Fig. 1. Effect of hydrophobicity of ion-pairing reagent on RP-HPLC retention behaviour of a positively charged model peptide mixture. Conditions: linear AB gradient (0.5% B min^{-1}) at a flow-rate of 0.3 ml/min, where Eluent A is 10 mM aq. phosphoric acid, TFA, PFPA or HFBA and Eluent B is the corresponding ion-pairing reagent concentration in acetonitrile; temperature, 25 °C. The sequences and denotions of the peptides are shown in Table 1.

Note that 10 mM TFA is equivalent to ∼0.08% TFA, i.e., within the 0.05–0.1% TFA concentration range commonly employed for peptides [4–6,10].

From Fig. 1 and Table 2, increasing counterion hydrophobicity (phosphate < TFA^- < $PFPA^-$ < $HFBA^-$) generally results, as expected [7–10], in increasing peptide retention time. There is also a general overall peak shape improvement with increasing peptide hydrophobicity due to increasing counterion hydrophobicity.

In addition, this effect of increasing counterion hydrophobicity is more marked the greater the positive charge on the peptides, i.e., +1 group < +3 group < +5 group. This effect is especially noticeable for the +5 group peptides, where early eluted peptides (e.g., 5a, 5e), in particular exhibited severe tailing and poor peak shape in the presence of 10 mM H_3PO_4. In addition, the relationship between counterion hydrophobicity and net positive charge on the peptides results in dramatic selectivity differences in the presence of the different ion-pairing reagents. Thus, in 10 mM H_3PO_4, peptides 5a and 5e are eluted first, i.e., under such conditions they are the most hydrophilic as expressed by RP-HPLC

Table 2
Effect of ion-pairing reagent hydrophobicity and concentration on retention times of +1, +3 and +5 peptides

Peptide[a]	Retention time (min)[b]											
	10 mM				20 mM				30 mM			
	H_3PO_4	TFA	PFPA	HFBA	H_3PO_4	TFA	PFPA	HFBA	H_3PO_4	TFA	PFPA	HFBA
1a	22.21	24.73	27.99	31.74	22.30	25.47	28.72	32.42	22.34	25.76	29.06	32.66
1b	24.81	27.07	30.16	33.73	24.83	27.83	30.87	34.35	24.79	27.95	31.19	34.57
1c	26.98	29.30	32.17	35.63	27.02	29.95	32.88	36.23	27.03	30.16	33.20	36.46
1d	30.61	32.98	35.60	39.01	30.63	33.48	36.26	39.62	30.64	33.80	36.68	39.85
3a	16.93	24.23	33.98	44.31	17.50	26.72	36.26	46.41	17.52	27.63	37.52	47.32
3b	18.81	26.07	35.60	45.47	19.25	28.56	37.90	47.59	19.29	29.55	39.02	48.55
3c	20.72	28.22	37.21	46.75	21.17	30.67	39.55	48.87	21.30	31.58	40.68	49.85
3d	23.42	30.80	39.44	48.67	23.64	33.11	41.75	50.79	23.76	34.08	42.89	51.81
5a	10.67	22.45	39.44	54.69	11.24	26.26	42.64	57.57	10.66	27.95	44.36	58.96
5e	14.87	27.07	42.64	56.62	15.48	30.67	45.91	59.51	15.42	32.54	47.50	60.87
5h	18.21	30.80	45.65	58.61	18.85	34.98	48.79	61.47	19.29	36.44	50.32	62.85
5j	22.82	36.38	49.46	61.44	23.64	39.80	52.61	64.43	23.76	41.26	54.20	65.94

[a] Peptide sequences shown in Table 1.
[b] RP-HPLC conditions, see Section 2.2.

retention time. In contrast, in the presence of 10 mM HFBA, they are amongst the latest peptides eluted, i.e., under such conditions they are the most hydrophobic when monitored in this way. Hence, as the hydrophobicity of the counterion increases, the relative hydrophobicity of the peptides are increasing in the order of +1 peptides < +3 peptides < +5 peptides, resulting in a relative change in peptide elution order with increasing counterion hydrophobicity and culminating in the excellent resolution of all 12 peptides in the presence of 10 mM HFBA, whereby the peptides are separated by charged groups and hydrophobicity within these groups.

A quantitative comparison of the relative effect on RP-HPLC of differently charged peptides may be obtained by considering the increase in retention times of the peptides in TFA, PFPA and HFBA relative to those obtained in H_3PO_4. Thus, taking one representative peptide from each charge group as an example: peptide 1a (+1 net charge) shows an increase in retention time of 2.52, 5.78 and 9.53 min, respectively, in 10 mM TFA, PFPA and HFBA relative to 10 mM H_3PO_4; peptide 3a (+3 net charge) shows an increase in retention time of 7.3, 17.05 and 27.38 min, respectively; and peptide 5a (+5 net charge) shows an increase in retention time of 11.78, 28.77 and 44.02 min, respectively. This general trend is also repeated for the remaining peptides in the three charged groups. In addition, in the presence of 10 mM ion-pairing reagent, the increase in peptide retention time per positive charge with increasing counterion hydrophobicity is similar for all three peptide groups: an average increase of 2.37, 2.45 and 2.51 min for the +1, +3 and +5 groups, respectively in 10 mM TFA; an average increase of 5.33, 5.53

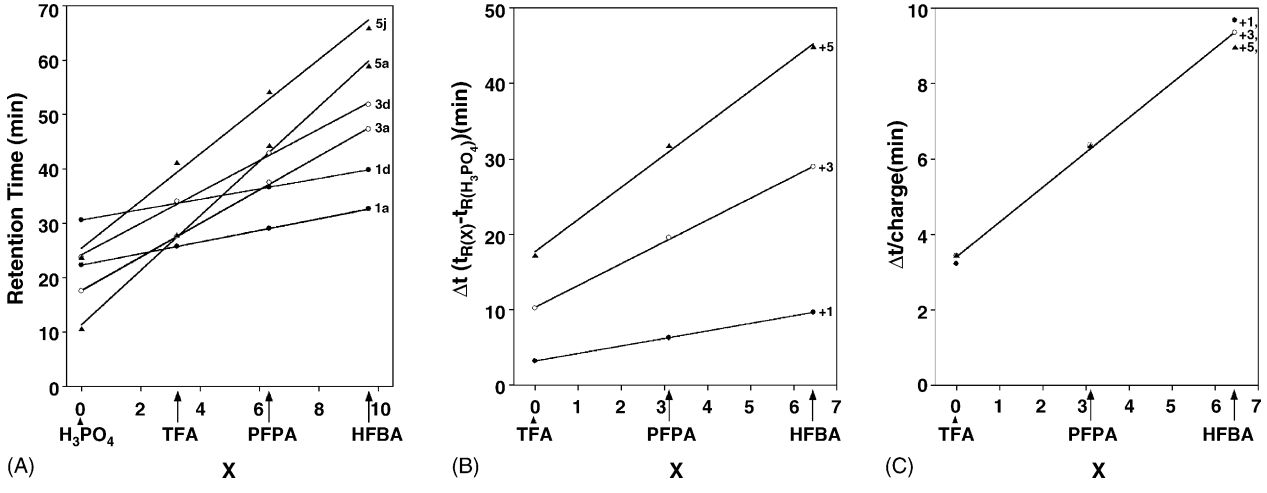

Fig. 2. Effect of hydrophobicity of ion-pairing reagent (30 mM) on RP-HPLC retention behaviour of a positively charged model peptide mixture. Panels (A) retention time vs. average increase in retention time per positively charged residue of representative peptides of the three groups. (B) Average increase in retention time in 30 mM TFA, PFPA and HFBA over that obtained in 30 mM H_3PO_4 (Table 3). (C) Average increase in retention time in 30 mM TFA, PFPA or HFBA over that obtained in 30 mM H_3PO_4 per positively charged residue. The average increase in retention time per positively charged residue was taken from the average values for the +1 group, i.e., 3.22 min for 30 mM TFA; 6.33 min for 30 mM PFPA; and 9.68 min for 30 mM HFBA. The sequences of the peptides are shown in Table 1. For RP-HPLC conditions, see Section 2.2.

and 5.53 min, respectively, in 10 mM PFPA; and an average increase of 8.88, 8.78 and 8.24 min, respectively, in 10 mM HFBA. Similar results were also obtained at concentrations of 20 and 30 mM.

Fig. 2 represents a graphical summary of the overall effect of increasing counterion hydrophobicity on peptide elution behaviour. This effect is expressed as that of the increase in retention times of two representative peptides of each peptide group (1a, 1d, +1 net charge; 3a, 3d, +3 net charge; 5a, 5j, +5 net charge) in 30 mM acid (panel A), the average increase in peptide retention time of all peptides within a group relative to phosphoric acid (30 mM acid) (panel B) and average increase in peptide retention time of all peptides within a group per net positive charge (panel C) over that obtained in 30 mM H_3PO_4 as the hydrophobicity of the counterion increases in the order $TFA^- < PFPA^- < HFBA^-$ (30 mM of each). From Fig. 2, panel A, the increase in peptide retention time with increasing counterion hydrophobicity is quite clear, together with the increasing magnitude of this effect with increasing net positive charge on the peptides. From Fig. 2, panel C, the essentially identical effect of increasing counterion hydrophobicity per net positive charge on the peptides is also highlighted. Such results are useful when attempting to predict the effect of varying counterion hydrophobicity during, for example, development of peptide separation protocols [10].

3.4. Effect of concentration of ion-pairing reagent on peptide elution behaviour

From Fig. 3, increasing TFA concentration generally results in increasing peptide retention time (Table 2) and improved peak shape, reflecting previous observations in our laboratory [12]. In addition, this effect of increasing TFA concentrations was more marked the greater the positive charge on the peptide, i.e., +1 group < +3 group < +5 group. From Fig. 3, it is perhaps easiest to visualize this latter result through observation of the effect of TFA concentration on peptides 5j (+5), 3c (+3) and 1c (+1). Thus, comparing the results for 10 mM TFA and 30 mM TFA, the effect of increasing TFA concentration on peptide peak shape/width is clearly most marked for peptide 5j compared to 3c and 1c. Note that, for this particular peptide mixture, resolution of all 12 peptides was never achieved over the concentration range of TFA employed.

In contrast to the results shown in Fig. 3, resolution of all 12 peptides was achieved in the presence of 30 mM PFPA (Fig. 4). In addition, only two pairs or one pair of peptides were co-eluted in 10 mM PFPA and 20 mM PFPA, respectively, still an improvement on average of the separations achieved in the presence of TFA (Fig. 3) with this particular peptide mixture. In a similar manner to the results achieved with TFA (Fig. 3), increasing PFPA concentration (Fig. 4) also generally results in increasing peptide retention time (Table 2) and sharpening of the peptide peaks, this effect again being more marked the greater the positive charge on the peptides. Indeed, the complete resolution of all 12 peptides

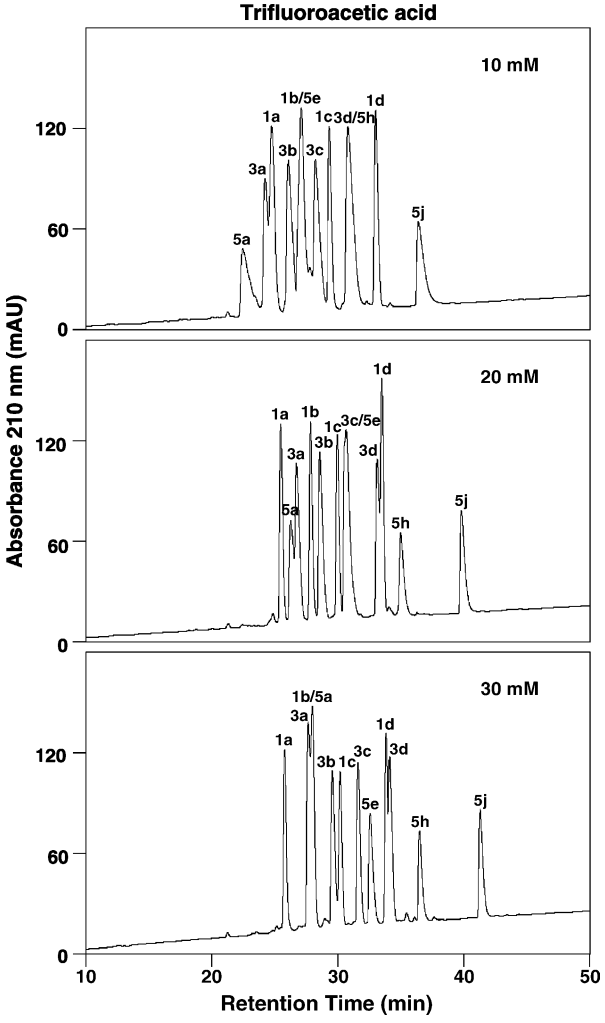

Fig. 3. Effect of TFA concentration on RP-HPLC retention behaviour of positively charged peptide mixture. Conditions: linear AB gradient (0.5% B min^{-1}) at a flow-rate of 0.3 ml/min, where Eluent A is 10, 20 or 30 mM aq. TFA and Eluent B is the corresponding TFA concentration in acetonitrile, temperature, 25 °C. The sequences and denotions of the peptides are shown in Table 1.

at 30 mM PFPA (Fig. 4) is due to the relationship between PFPA concentration and net positive charge on the peptides, i.e., the greater the positive charge (+5 > +3 > +1), the greater the change in peptide retention time, with increasing PFPA concentration until, in a similar manner to the 10 mM HFBA results (Fig. 1), the peptides are separated by charged groups (+1 < +3 < +5) and hydrophobicity within these groups. Although, as noted above, similar effects on the retention behaviour of the three peptide groups were observed with increasing TFA concentration (Fig. 1), the hydrophobicity of the TFA^- counterion was not of a great enough magnitude to achieve this separation by charged groups (+1 < +3 < +5), even at a concentration of 30 mM. Note that similar effects of ion-pairing reagent concentration on peptide elution behaviour were observed for 10–30 mM HFBA. However, since an excellent separation of all 12 peptides had already been achieved at just 10 mM HFBA (Fig. 1), the peptide

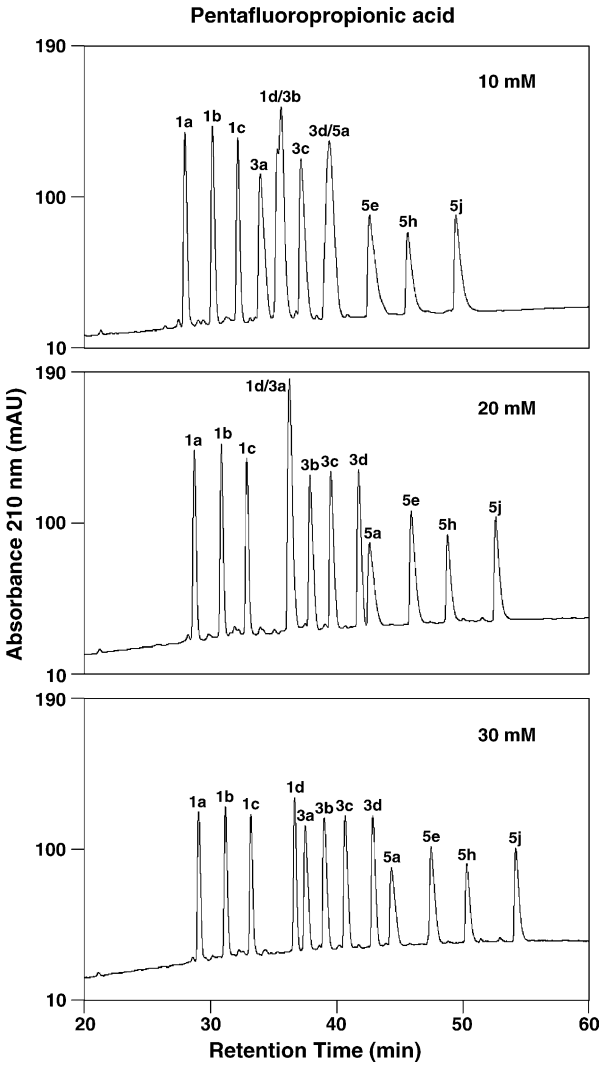

Fig. 4. Effect of PFPA concentration on RP-HPLC retention behaviour of positively charged peptide mixture. Conditions: linear AB gradient (0.5% B/min) at a flow-rate of 0.3 ml/min, where Eluent A is 10, 20 or 30 mM aq. PFPA and Eluent B is corresponding PFPA concentration in acetonitrile, temperature, 25 °C. The sequences and denotions of the peptides are shown in Table 1.

elution profiles for 20 and 30 mM HFBA are not shown here.

It is interesting to note that, although the effect of increasing counterion hydrophobicity on peptide retention time was quite dramatic (Figs. 1 and 2), the effect of increasing counterion concentration, at least over the 10–30 mM range, appears not so clear cut, where the response of all three groups of peptides to increasing concentrations of H_3PO_4, TFA, PFPA and HFBA were similar despite the increase in counterion hydrophobicity of phosphate < TFA^- < $PFPA^-$ < $HFBA^-$ (Table 2). This phenomenon is investigated in greater detail in a companion study [25] which investigates the effect on peptide retention behaviour of anionic ion-pairing reagent concentration over a much wider concentration range (1–60 mM) compared to the present study.

3.5. Effect of concentration of ion-pairing reagent on peptide resolution

Table 3 reports the effect of increasing concentration (10–30 mM) of the four ion-pairing reagents on the resolution of representative peptide pairs 1a/1b and 5h/5j, i.e., resolution of peptides with the same net positive charge (+1 and +5, respectively). From Table 3, the retention time differences (Δt) between the peptides within the two peptide pairs remained essentially identical throughout the concentration range of the four ion-pairing reagents, i.e., Δt values were independent of counterion concentration. However, increasing counterion concentration produces a significant decrease in peptide peak width ($W_{1/2}$), this decrease being particularly dramatic in the case of phosphoric acid compared to the three perfluorinated acids. Thus, peptides 1a and 1b (+1 net charge) decreased from peak width values of 0.474 and 0.478, respectively, at 10 mM H_3PO_4 to values of 0.286 and 0.285, respectively at 30 mM H_3PO_4; even more dramatically, peptides 5h and 5j (+5 net charge) decreased from peak widths of 0.994 and 1.103, respectively, at 10 mM H_3PO_4 to values of 0.398 and 0.299, respectively, at 30 mM H_3PO_4. In contrast, taking HFBA as representative of the perfluorinated acids, peptides 1a and 1b decreased from 0.272 and 0.260, respectively, in 10 mM HFBA to 0.251 and 0.258, respectively in 30 mM HFBA; peptides 5h and 5j decreased from peak widths of 0.478 and 0.518, respectively, in 10 mM HFBA to 0.340 and 0.339, respectively, in 30 mM HFBA. All three perfluorinated acids showed similar magnitudes of peak width values over the concentration range employed in this study. Note that, at the highest concentration (30 mM) of reagents used in the present study, peak width values obtained for the +5 peptides were higher than those generally obtained for the +1 peptides. This trend was also observed for the +3 group peptides, whose peak width values at a concentration of 30 mM ion-pairing reagent were generally intermediate between those obtained for the +1 and +5 groups. In addition, the ranges of peak widths for the +1 peptides over the 10–30 mM concentration range of ion-pairing reagents was always less than those observed for the +5 peptides. For example, for peptide 1a (+1 net charge), there was a 39.7% decrease in peak width between 10 mM H_3PO_4 and 30 mM H_3PO_4; and there was just a 7.6% decrease in peak width between 10 mM PFPA and 30 mM PFPA, chosen as representative of the perfluorinated acid series. In contrast, for peptide 5h (+5 net charge), there was a 60.0% decrease in peak width between 10 mM H_3PO_4 and 30 mM H_3PO_4; and there was a 33.8% decrease in peak width between 10 mM PFPA and 30 mM PFPA.

Finally, with Δt values between peptides 1a and 1b and between peptides 5h and 5j being essentially independent of counterion concentrations but with $W_{1/2}$ decreasing with increasing counterion concentration, it would be expected that peptide resolution would concomitantly increase with increasing counterion concentration, as was indeed observed (Table 3). The relative increase in resolution for the +5 peptides (5h/5j) with increasing counterion concentration was

Table 3
Effect of ion-pairing reagents on resolution of peptide pairs 1a/1b (+1 net charge) and 5h/5j (+5 net charge)

Condition	1a/1b				5h/5j			
	Δt	$W_{1/2}$ (1a)[a]	$W_{1/2}$ (1b)	R_s[b]	Δt	$W_{1/2}$ (5h)	$W_{1/2}$ (5j)	R_s
10 mM H_3PO_4	2.56	0.474	0.478	3.16	4.78	0.994	1.103	2.68
20 mM H_3PO_4	2.53	0.333	0.335	4.45	4.81	0.605	0.675	4.42
30 mM H_3PO_4	2.39	0.286	0.285	4.92	4.91	0.398	0.299	8.27
10 mM TFA	2.39	0.292	0.283	4.89	4.88	0.506	0.509	5.65
20 mM TFA	2.38	0.254	0.245	5.6	4.89	0.349	0.36	8.1
30 mM TFA	2.35	0.248	0.238	5.69	4.82	0.302	0.325	9.05
10 mM PFPA	2.18	0.288	0.287	4.45	3.82	0.532	0.524	4.26
20 mM PFPA	2.14	0.266	0.265	4.75	3.83	0.385	0.413	5.64
30 mM PFPA	2.13	0.266	0.261	4.81	3.89	0.352	0.379	6.25
10 mM HFBA	1.99	0.272	0.26	4.4	2.83	0.478	0.518	3.35
20 mM HFBA	1.93	0.274	0.259	4.25	2.96	0.403	0.406	4.3
30 mM HFBA	1.89	0.251	0.258	4.37	3.1	0.34	0.339	5.38

[a] $W_{1/2}$ (X) denote the width of half bandwidth of the observed peptide X peak.
[b] R_s denote the resolution between the peaks of two peptides, calculated as described in the text.

always greater than observed for the +1 peptides (1a/1b) as one would expect given the above observation concerning relative peak width ranges of the differently charged peptides over the ion-pairing reagent concentration range studied.

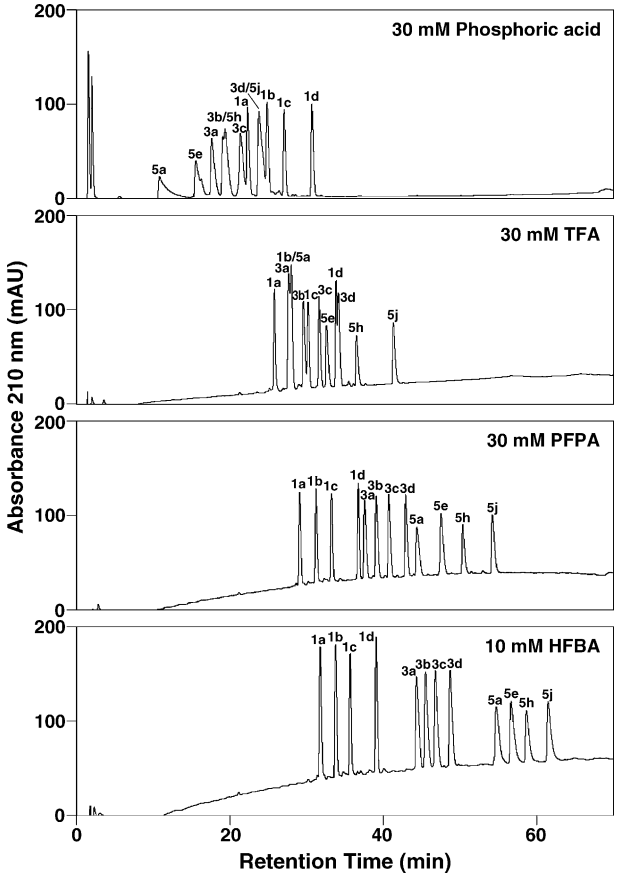

Fig. 5. Optimum RP-HPLC separation of positively charged model peptide mixture in each ion-pairing regent system. Conditions: linear AB gradient (0.5% B min^{-1}) at a flow-rate of 0.3 ml/min, where Eluent A is 30 mM aq. phosphoric acid, TFA, PFPA or 10 mM aq. HFBA and Eluent B is the corresponding ion-pairing reagent concentration in acetonitrile; temperature, 25 °C. The sequences and denotions of the peptides are shown in Table 1.

Thus, for example, resolution of peptide pair 1a/1b improved 1.56-fold in the H_3PO_4 system and just 1.08-fold in the PFPA system. In contrast, resolution of peptide pair 5h/5j improved by 3.09-fold in the H_3PO_4 system and 1.47-fold in the PFPA system.

Fig. 5 now represents a comparison of the optimum separation of this mixture of model peptides by each of the four ion-pairing reagents. Clearly, the best separation was obtained in the presence of 10 mM HFBA, although baseline resolution of all 12 peptides was obtained in 30 mM PFPA. Such complete separation was never obtained in phosphoric acid or TFA for this particular peptide mixture. Indeed, the use of phosphoric acid appears to be inappropriate for the analysis of highly charged peptides (>+3) due to severe peak tailing, particularly for early eluted peptides. However, Fig. 5 demonstrates well the range of options to researchers in terms of potential efficacy of variations in counterion hydrophobicity and/or concentration to optimize peptide separation.

4. Conclusions

The present study has investigated the effect of varying hydrophobicity and concentration of anionic ion-pairing reagents (phosphoric acid, TFA, PFPA, HFBA) on RP-HPLC of a mixture of synthetic model peptides, containing peptides of +1, +3 or +5 net charge. Clear selectivity differences were observed depending on the nature and concentration of the ion-pairing reagent, with overall separation of the 12-peptide mixture improving with increasing hydrophobicity (phosphate < TFA^- < $PFPA^-$ < $HFBA^-$) and/or concentration of the counterion. Peptide peak widths decreased with a concomitant increase in peptide resolution with increasing counterion concentration. Optimum separation of the 12 peptides was achieved with just 10 mM HFBA, whereby the peptides were separated by charged groups (+1 < +3 < +5) and hydrophobicity within these groups, i.e., the resolution obtained with 10 mM HFBA is a mixed effect of reagent hydrophobicity and concentration together with peptide

charge. The predictable nature of the effects of various ion-pairing reagents on peptide retention behaviour should prove useful for the rational design of peptide separation protocols.

Acknowledgement

This work was supported by an NIH grant (RO1 GM61855) to Robert S. Hodges.

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