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Journal of Chromatography A, 1080 (2005) 49–57
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

The perchlorate anion is more effective than the trifluoroacetate anion as an ion-pairing reagent for reversed-phase chromatography of peptides

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Available online 29 March 2005

Abstract

The addition of salts, specifically sodium perchlorate ($NaClO_4$), to mobile phases at acidic pH as ion-pairing reagents for reversed-phase high-performance liquid chromatography (RP-HPLC) has been generally overlooked. To demonstrate the potential of $NaClO_4$ as an effective anionic ion-pairing reagent, we applied RP-HPLC in the presence of 0–100 mM sodium chloride (NaCl), sodium trifluoroacetate (NaTFA) or $NaClO_4$ to two mixtures of synthetic 18-residue peptides: a mixture of peptides with the same net positive charge (+4) and a mixture of four peptides of +1, +2, +3 and +4 net charge. Interestingly, the effect of increasing $NaClO_4$ concentration on increasing peptide retention times and selectivity changes was more dramatic than that of either NaCl or NaTFA, with the order of increasing anion effectiveness being $Cl^- \ll TFA^- < ClO_4^-$. Such effects were more marked when salt addition was applied to eluents containing 10 mM phosphoric acid (H_3PO_4) compared to 10 mM trifluoroacetic acid (TFA) due to the lesser starting anion hydrophobicity of the former mobile phase (containing the phosphate ion) compared to the latter (containing the TFA^- ion).

Keywords: Peptides; Anions; RP-HPLC; Perchlorate; Trifluoroacetate

1. Introduction

Peptides are charged molecules at most pH values, with basic (positively charged) character dominating at low pH, due to protonation of any potentially negatively charged acidic side-chains. Differences in the hydrophilicity/hydrophobicity of peptides at acidic pH can be maximized through variations in the choice of anionic ion-pairing reagent, e.g., phosphoric acid and trifluoroacetic acid (TFA), where the resolving power of the corresponding counterions (phosphate and trifluoroacetate, respectively) during reversed-phase high-performance liquid chromatography (RP-HPLC) is effected through interaction with the positively charged (basic) residues of the peptide [1–4]. The observed effect on peptide retention time during RP-HPLC depends on the hydrophobicity of the anionic counterion as well as the number of positively charged groups in the peptide [1].

TFA remains the dominant hydrophobic ion-pairing reagent for peptide separations [2–4], although more hydrophilic or hydrophobic reagents such as phosphoric acid [1–10] and heptafluorobutyric acid (HFBA) [1,2,4,11–15], respectively, are also employed for specific peptide applications. However, we believe that the value of addition of salts to mobile phases at acidic pH as ion-pairing reagents has been generally overlooked in recent years. Addition of salts (generally 50–100 mM) to mobile phases over a pH range ~4–7 has generally been designed, for silica-based packings, to suppress negatively charged silanol interactions with positively charged solutes [2,4,16–24], with potential selectivity effects tending to be a secondary consideration. However, our laboratory has already demonstrated how salt (specifically sodium perchlorate) addition often offers gains in peptide selectivity at low pH [25] and now wished to investigate the potential of sodium perchlorate, as well as other salts, as anionic ion-pairing reagents at low pH. Thus, we applied RP-HPLC in the presence of 0–100 mM sodium chloride (NaCl), sodium trifluoroacetate (NaTFA) or sodium perchlorate ($NaClO_4$) to

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doi:10.1016/j.chroma.2005.02.063

two mixtures of model 18-residue peptides, one mixture containing six peptides with the same net positive charge (+4) and the other containing four peptides with varying net charge (+1, +2, +3, +4). We believe that these, and continuing, studies will add further to our knowledge of the best approaches to effective separation of complex peptide mixtures.

2. Experimental

2.1. Materials

Reagent-grade phosphoric acid (H_3PO_4) was obtained from Caledon Laboratories (Georgetown, Ontario, Canada). TFA was obtained from Hydrocarbon Products (River Edge, NJ, USA); NaCl, NaTFA, and $NaClO_4$ were obtained from Sigma–Aldrich (St. Louis, MO, 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 mM aq. H_3PO_4 or 10 mM aq. TFA and eluent B was 50% aq. acetonitrile containing 10 mM H_3PO_4 or 10 mM TFA, respectively, and both eluents also containing 0, 5, 10, 25, 50 or 100 mM, NaCl, NaTFA or $NaClO_4$; runs were carried out at 25 °C.

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 [26]. 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).

3. Results and discussion

3.1. Design of synthetic model peptides

We have designed and synthesized two groups of model peptides exhibiting variations in hydrophobicity and/or net positive charge. From Table 1, peptides 1–6 represent a group of six peptides with the same net charge (+4, due to the presence of four lysine residues). Within these peptides, hydrophobicity decreases only subtly between each adjacent peptide (peptide 6 < 5 < 4 < 3 < 2 < 1) due to just single substitutions of glutamine in place of glutamic acid, i.e., between each adjacent peptide, there is a single carboxyl group to amide group substitution. Peptides 6–9 represent a group of four peptides varying in net positive charge through the presence of four lysine residues (peptide 6; +4), three lysine residues (peptide 7; +3), two lysine residues (peptide 8; +2) or one lysine residue (peptide 9; +1). The presence of five glycine residues located throughout the sequence ensured negligible secondary structure for these peptides [27,28], i.e., they have a "random coil" conformation, to avoid complications in data interpretation due to selectivity differences in peptide RP-HPLC retention behaviour arising from conformational variations [10,29].

Table 1
Sequences of peptides used in this study

Peptides	Sequence[a]	Number of positive charges
1	Ac-KLKKGGLKGELGGEELEE-amide	4
2	Ac-KLKKGGLKGELGGEELE**Q**-amide	4
3	Ac-KLKKGGLKGELGGEEL**QQ**-amide	4
4	Ac-KLKKGGLKGELGGE**Q**L**QQ**-amide	4
5	Ac-KLKKGGLKGELGG**QQ**L**QQ**-amide	4
6	Ac-KLKKGGLKG**Q**LGG**QQ**L**QQ**-amide	4
7	Ac-KLKKGGL**A**GELGGEELEE-amide	3
8	Ac-KLK**A**GGL**A**GELGGEELEE-amide	2
9	Ac-KL**AA**GGL**A**GELGGEELEE-amide	1

[a] Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide; the changes in the sequence compared to peptide 1 are shown in bold.

Table 2
Effect of sodium perchlorate concentration in 10 mM phosphoric acid

Peptide[a]	Retention time (min)[b]						5 mM		10 mM		25 mM		50 mM		100 mM	
	0 mM	5 mM	10 mM	25 mM	50 mM	100 mM	Δt[c]	$\Delta t/N$[d]	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$
6 (+4)	26.55	32.90	35.25	41.00	41.58	42.14	6.35	1.59	8.70	2.17	14.45	3.61	15.03	3.76	15.59	3.90
5 (+4)	27.85	34.41	36.81	42.64	43.19	43.72	6.56	1.64	8.96	2.24	14.79	3.70	15.34	3.84	15.87	3.97
4 (+4)	28.82	35.39	37.79	43.60	44.12	44.62	6.58	1.64	8.97	2.24	14.78	3.69	15.30	3.83	15.80	3.95
3 (+4)	29.85	36.49	38.91	44.73	45.23	45.71	6.65	1.66	9.06	2.27	14.88	3.72	15.39	3.85	15.86	3.96
2 (+4)	30.80	37.51	39.93	45.75	46.22	46.67	6.71	1.68	9.13	2.28	14.95	3.74	15.42	3.86	15.87	3.97
1 (+4)	31.71	38.33	40.74	46.52	46.98	47.40	6.62	1.66	9.04	2.26	14.82	3.70	15.27	3.82	15.69	3.92
Average							6.58	1.64	8.98	2.24	14.78	3.69	15.29	3.82	15.78	3.94
1 (+4)	31.56	38.41	40.75	44.69	46.73	46.96	6.86	1.71	9.20	2.30	13.13	3.28	15.17	3.79	15.40	3.85
7 (+3)	37.08	42.38	44.07	47.14	48.74	48.71	5.30	1.77	6.99	2.33	10.06	3.35	11.66	3.89	11.63	3.88
8 (+2)	42.18	45.51	46.47	48.68	49.94	49.73	3.33	1.67	4.30	2.15	6.50	3.25	7.77	3.88	7.56	3.78
9 (+1)	46.72	48.12	48.42	49.83	50.78	50.42	1.40	1.40	1.71	1.71	3.11	3.11	4.07	4.07	3.70	3.70
Average								1.64		2.12		3.25		3.91		3.80

[a] Peptide sequences shown in Table 1.
[b] RP-HPLC conditions, see Section 2.2.
[c] $\Delta t = t_R$ of peptide at a particular $NaClO_4$ concentration minus t_R in the absence of salt.
[d] N = number of positively charged residues in peptide.

3.2. Effect of salts on RP-HPLC retention behaviour of peptides with identical net positive charge

Tables 2–4 (top panels) present retention time data obtained from the effect on retention times of peptides 1–6 (+4 net charge) of the addition of $NaClO_4$ (Table 2), NaTFA (Table 3) or NaCl (Table 4), over a concentration range of 5–100 mM, to a mobile phase containing 10 mM H_3PO_4. Thus, with no salt present, the sole anionic counterion is the hydrophilic phosphate anion. The use of $NaClO_4$ in these studies was prompted by a number of considerations, including common use as a reagent in RP-HPLC at both neutral [16,18,22] and acidic [8,16,25,30,31] pH values to improve peak shape and its high solubility in aqueous acetonitrile eluents, even at relatively high concentrations of this organic modifier [32–34]. In addition, the historical use of sodium perchlorate as an anionic, hydrophilic ion-pairing reagent [35] has been generally superceded in recent years by its employment solely to suppress non-specific ionic interactions between positively charged peptide groups and any negatively charged free silanols in silica-based reversed-phase matrices, particularly at neutral pH [2,4,16,18,22,24]. We have previously shown that the addition of sodium perchlorate at a fixed and high (100 mM) concentration produces useful selectivity effects for peptide separations at low pH [24]. The present study now reexamines the potential worth of the perchlorate anion as an ion-pairing reagent in comparison with the commonly utilized TFA^- anion when added to the mobile phase as sodium salts. The addition of NaTFA to the mobile phase reflects the dominance of the trifluoroacetate

Table 3
Effect of sodium trifluoroacetate concentration in 10 mM phosphoric acid

Peptide[a]	Retention time (min)[b]						5 mM		10 mM		25 mM		50 mM		100 mM	
	0 mM	5 mM	10 mM	25 mM	50 mM	100 mM	Δt[c]	$\Delta t/N$[d]	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$
6 (+4)	26.55	30.95	33.09	35.70	37.89	39.47	4.40	1.10	6.54	1.63	9.15	2.29	11.34	2.83	12.92	3.23
5 (+4)	27.85	32.36	34.54	37.19	39.40	41.03	4.51	1.13	6.69	1.67	9.34	2.33	11.55	2.89	13.18	3.29
4 (+4)	28.82	33.29	35.47	38.10	40.30	41.90	4.47	1.12	6.65	1.66	9.28	2.32	11.48	2.87	13.08	3.27
3 (+4)	29.85	34.30	36.50	39.13	41.33	42.95	4.46	1.11	6.65	1.66	9.28	2.32	11.49	2.87	13.10	3.28
2 (+4)	30.80	35.22	37.43	40.07	42.27	43.89	4.42	1.11	6.63	1.66	9.27	2.32	11.47	2.87	13.09	3.27
1 (+4)	31.71	35.88	38.07	40.71	42.90	44.51	4.18	1.04	6.37	1.59	9.01	2.25	11.20	2.80	12.80	3.20
Average							4.41	1.10	6.59	1.65	9.22	2.31	11.42	2.85	13.03	3.26
1 (+4)	31.56	35.80	37.91	40.76	42.77	44.43	4.24	1.06	6.35	1.59	9.21	2.30	11.21	2.80	12.88	3.22
7 (+3)	37.08	40.28	41.87	44.04	45.60	46.88	3.20	1.07	4.79	1.60	6.96	2.32	8.52	2.84	9.80	3.27
8 (+2)	42.18	43.94	44.97	46.43	47.57	48.54	1.77	0.88	2.79	1.40	4.25	2.13	5.40	2.70	6.37	3.18
9 (+1)	46.72	47.12	47.63	48.44	49.17	49.85	0.41	0.41	0.92	0.92	1.72	1.72	2.45	2.45	3.13	3.13
Average								0.85		1.37		2.12		2.70		3.20

[a] Peptide sequences shown in Table 1.
[b] RP-HPLC conditions, see Section 2.2.
[c] $\Delta t = t_R$ of peptide at a particular NaTFA concentration minus t_R in the absence of salt.
[d] N = number of positively charged residues in peptide.

Table 4
Effect of sodium chloride concentration in 10 mM phosphoric acid

Peptide[a]	Retention time (min)[b]						5 mM		10 mM		25 mM		50 mM		100 mM	
	0 mM	5 mM	10 mM	25 mM	50 mM	100 mM	Δt[c]	$\Delta t/N$[d]	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$
6 (+4)	26.55	27.60	28.48	29.65	30.53	31.49	1.05	0.26	1.93	0.48	3.10	0.78	3.98	0.99	4.94	1.23
5 (+4)	27.85	28.96	29.89	31.13	32.04	33.00	1.11	0.28	2.04	0.51	3.28	0.82	4.19	1.05	5.15	1.29
4 (+4)	28.82	29.93	30.86	32.08	33.00	33.93	1.11	0.28	2.04	0.51	3.26	0.81	4.18	1.04	5.11	1.28
3 (+4)	29.85	30.95	31.91	33.14	34.08	35.01	1.10	0.27	2.06	0.52	3.30	0.82	4.24	1.06	5.16	1.29
2 (+4)	30.80	31.89	32.88	34.14	35.11	36.03	1.09	0.27	2.08	0.52	3.34	0.83	4.31	1.08	5.23	1.31
1 (+4)	31.71	32.60	33.59	34.83	35.82	36.72	0.89	0.22	1.89	0.47	3.13	0.78	4.11	1.03	5.01	1.25
Average							1.06	0.26	2.01	0.50	3.23	0.81	4.17	1.04	5.10	1.28
1 (+4)	31.56	32.44	33.08	34.36	35.39	36.44	0.88	0.22	1.52	0.38	2.81	0.70	3.84	0.96	4.88	1.22
7 (+3)	37.08	37.89	38.33	39.24	39.98	40.78	0.81	0.27	1.25	0.42	2.16	0.72	2.90	0.97	3.70	1.23
8 (+2)	42.18	42.52	42.75	43.24	43.68	44.23	0.35	0.17	0.58	0.29	1.07	0.53	1.51	0.75	2.05	1.03
9 (+1)	46.72	46.63	46.65	46.79	46.96	47.26	−0.09	−0.09	−0.06	−0.06	0.07	0.07	0.24	0.24	0.54	0.54
Average								0.14		0.26		0.51		0.73		1.00

[a] Peptide sequences shown in Table 1.
[b] RP-HPLC conditions, see Section 2.2.
[c] $\Delta t = t_R$ of peptide at a particular NaCl concentration minus t_R in the absence of salt.
[d] N = number of positively charged residues in peptide.

ion (TFA^-) as the continuing anionic, hydrophobic counterion of choice for peptide separations in RP-HPLC. Finally, the addition of NaCl represents a useful standard salt, with the chloride ion (Cl^-) providing a comparison to perchlorate (ClO_4^-) and the more hydrophobic TFA^- anion. All three salts are sodium salts to assure any effects of the salts on peptide retention behaviour may be assigned solely to the anionic counterion.

From Tables 2–4, it is clear that all three salts produce an increase in peptide retention time with increasing salt concentration, albeit to differing extents. These differences are clearly shown in Fig. 1 (left panel) which shows the effect of varying salt concentration on the retention time of peptides 1–6 relative to these values in the absence of salt,

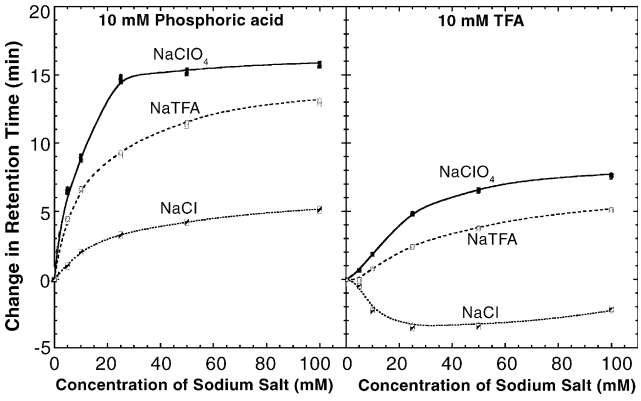

Fig. 1. Effect of salt concentration on RP-HPLC of six model peptides with the same net positive charge (+4) relative to that obtained in the absence of salt. Conditions: linear AB gradient (0.5% acetonitrile/min) at a flow-rate of 0.3 ml/min, where eluent A was water and eluent B was 50% aq. acetonitrile, both eluents containing 10 mM H_3PO_4 (left panel) or 10 mM TFA (right panel) and both eluents also containing 5, 10, 25, 50 mM or 100 mM NaCl, NaTFA or $NaClO_4$; runs were carried out at 25 °C. The sequences of the peptides (peptides 1–6) are shown in Table 1.

i.e., a normalization of the retention time data relative to those obtained in just 10 mM H_3PO_4. The relative effectiveness of the salts on increasing peptide retention time are $NaCl \ll NaTFA < NaClO_4$. This greater effectiveness of $NaClO_4$ compared to NaTFA is a particularly interesting observation when one considers that the TFA^- counterion is more hydrophobic than ClO_4^-, generally considered a relatively hydrophilic counterion, i.e., these results suggest that $NaClO_4$ is a more effective ion-pairing reagent than NaTFA, despite such a ranking of counterion hydrophobicity. For each salt, the effects of increasing concentration are essentially identical for these six peptides (all +4 net charge), i.e., assuming the observed effects are due to ion-pairing of the negatively charged salt anions with the four positively charged lysine residues in the peptides, the effect per positively charged residue is the same. The Cl^- ion, being the most hydrophilic, likely exerts its effect through neutralization of the positively charged lysine residues, thus decreasing the overall hydrophilicity of the peptides and resulting in increased retention times, i.e., there is no further enhancement of peptide retention time beyond that of simple neutralization of their positively charged character. In contrast, the TFA^- ion not only neutralizes the positive charges on the peptides but its more hydrophobic nature, relative to Cl^-, also enhances the hydrophobic character of the peptides, with concomitant considerable increases in retention times relative to NaCl addition. Following the initial rapid increase in peptide retention times at lower salt concentrations, this increase in retention time decreases gradually with increasing salt concentration until a plateau is effectively reached between 30 and 50 mM salt, indicating increasing saturation of the four lysine residues in the peptides by high concentrations of salt anions. In a previous study [24], it was noted that an increase in TFA concentration of just 44 mM (6–50 mM) in a solely acidic mobile phase increased retention times of pos-

Table 5
Effect of sodium perchlorate concentration in 10 mM TFA on peptide retention behaviour

Peptide[a]	Retention time (min)[b]						5 mM		10 mM		25 mM		50 mM		100 mM	
	0 mM	5 mM	10 mM	25 mM	50 mM	100 mM	Δt[c]	$\Delta t/N$[d]	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$
6 (+4)	34.57	35.22	36.41	39.32	41.04	42.17	0.65	0.16	1.83	0.46	4.75	1.19	6.47	1.62	7.60	1.90
5 (+4)	36.06	36.72	37.91	40.90	42.64	43.76	0.67	0.17	1.85	0.46	4.84	1.21	6.58	1.64	7.70	1.93
4 (+4)	37.01	37.67	38.84	41.84	43.56	44.66	0.66	0.17	1.84	0.46	4.83	1.21	6.55	1.64	7.65	1.91
3 (+4)	38.07	38.74	39.92	42.94	44.65	45.74	0.67	0.17	1.85	0.46	4.87	1.22	6.58	1.64	7.67	1.92
2 (+4)	39.06	39.72	40.89	43.94	45.60	46.67	0.66	0.16	1.83	0.46	4.88	1.22	6.54	1.64	7.61	1.90
1 (+4)	39.84	40.50	41.66	44.72	46.26	47.31	0.65	0.16	1.82	0.45	4.87	1.22	6.42	1.60	7.47	1.87
Average							0.66	0.17	1.84	0.46	4.84	1.21	6.52	1.63	7.62	1.90

[a] Peptide sequences shown in Table 1.
[b] RP-HPLC conditions, see Section 2.2.
[c] $\Delta t = t_R$ of peptide at a particular salt concentration minus t_R in the absence of salt.
[d] N = number of positively charged residues in peptide.

itively charged peptides to a similar extent as the addition of 100 mM $NaClO_4$ to 20 mM H_3PO_4. Thus, in this previous study, two very different mobile phase systems were being compared, i.e., an increase in acid concentration (TFA) versus the addition of a substantial amount of salt ($NaClO_4$) to a different acidic mobile phase (20 mM H_3PO_4). The present study represents a step-by-step comparison of the effect of varying concentrations of sodium salts of anions of interest in the same acidic mobile phase (10 mM H_3PO_4 or 10 mM TFA) for a more exact and valid comparison of relative anion effectiveness for peptide separations. Note that the results of the previous study [24] can be readily understood based on the results summarized in Fig. 1, which clearly show that the effectiveness of any added anion is dependent on the type and concentration of the anion in the buffer (the latter representing starting conditions prior to the addition of the selected anion). In addition, pH affects the concentration of the anion in the buffer and temperature affects the efficiency of ion-pair formation (the relevant results in the previous study [24] were obtained at 65 °C, whilst those of the present study were obtained at 25 °C). Thus, all these variables must be taken into consideration in understanding the final outcome and in making comparisons with other results.

An explanation for the observation that $NaClO_4$ is more effective than NaTFA in enhancing peptide hydrophobicity may lie in the strong chaotropic properties of the ClO_4^- anion which competes less effectively for nearby water molecules than does bulk water and is therefore dehydrated much more readily than ions such as Cl^-, which is approximately neutral on the Hofmeister scale (i.e., neither a kosmotrope nor a chaotrope) [36] and TFA^-. Strong ion-pairing reactions require the exclusion of water molecules from the interaction between the positively and negatively charged species, i.e., the anions used in the present study must be dehydrated to form the ion-pair with the lysine residues of the peptides. A much more readily dehydrated ClO_4^- anion compared to TFA^- could explain the greater effectiveness of $NaClO_4^-$ as an ion-pairing reagent over that of TFA^-, even though TFA^- is hydrophobic, i.e., the hydrophilic ClO_4^- anion is more efficient than TFA^- at ion-pairing with the positively charged groups in the peptides.

Tables 5–7 now show the effect of increasing salt concentration on the retention times of peptides 1–6 following addition of up to 100 mM NaCl (Table 5), NaTFA (Table 6) or $NaClO_4$ (Table 7) to 10 mM TFA. From Fig. 1 (right panel), the order of counterion effectiveness on peptide retention time is identical to that obtained with 10 mM H_3PO_4 (Fig. 1, left panel), i.e., $Cl^- \ll TFA^- < ClO_4^-$. However, the magnitude of the effect was now less with this 10 mM TFA mobile phase system. Indeed, there was an initial significant decline in pep-

Table 6
Effect of sodium trifluoroacetate concentration in 10 mM TFA on peptide retention behaviour

Peptide[a]	Retention time (min)[b]						5 mM		10 mM		25 mM		50 mM		100 mM	
	0 mM	5 mM	10 mM	25 mM	50 mM	100 mM	Δt[c]	$\Delta t/N$[d]	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$
6 (+4)	34.57	34.59	35.35	36.98	38.33	39.61	0.01	0.00	0.77	0.19	2.41	0.60	3.76	0.94	5.03	1.26
5 (+4)	36.06	36.06	36.84	38.50	39.87	41.22	0.00	0.00	0.78	0.19	2.44	0.61	3.81	0.95	5.16	1.29
4 (+4)	37.01	37.00	37.77	39.41	40.77	42.12	−0.01	0.00	0.76	0.19	2.41	0.60	3.76	0.94	5.12	1.28
3 (+4)	38.07	38.04	38.83	40.47	41.82	43.20	−0.03	−0.01	0.76	0.19	2.40	0.60	3.75	0.94	5.13	1.28
2 (+4)	39.06	38.97	39.79	41.44	42.79	44.18	−0.09	−0.02	0.73	0.18	2.38	0.59	3.73	0.93	5.12	1.28
1 (+4)	39.84	39.66	40.56	42.17	43.50	44.88	−0.19	−0.05	0.71	0.18	2.33	0.58	3.66	0.91	5.04	1.26
Average							−0.05	−0.01	0.75	0.19	2.39	0.60	3.74	0.94	5.10	1.27

[a] Peptide sequences shown in Table 1.
[b] RP-HPLC conditions, see Section 2.2.
[c] $\Delta t = t_R$ of peptide at a particular salt concentration minus t_R in the absence of salt.
[d] N = number of positively charged residues in peptide.

Table 7
Effect of sodium chloride concentration in 10 mM TFA on peptide retention behaviour

Peptide[a]	Retention time (min)[b]						5 mM		10 mM		25 mM		50 mM		100 mM	
	0 mM	5 mM	10 mM	25 mM	50 mM	100 mM	Δt[c]	$\Delta t/N$[d]	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$	Δt	$\Delta t/N$
6 (+4)	34.57	34.06	32.42	31.30	31.34	32.19	−0.52	−0.13	−2.15	−0.54	−3.28	−0.82	−3.24	−0.81	−2.39	−0.60
5 (+4)	36.06	35.59	33.87	32.73	32.79	33.77	−0.47	−0.12	−2.19	−0.55	−3.33	−0.83	−3.27	−0.82	−2.29	−0.57
4 (+4)	37.01	36.56	34.79	33.63	33.68	34.74	−0.45	−0.11	−2.22	−0.55	−3.38	−0.85	−3.33	−0.83	−2.27	−0.57
3 (+4)	38.07	37.64	35.83	34.64	34.70	35.87	−0.43	−0.11	−2.25	−0.56	−3.43	−0.86	−3.37	−0.84	−2.20	−0.55
2 (+4)	39.06	38.63	36.77	35.58	35.65	36.92	−0.43	−0.11	−2.29	−0.57	−3.48	−0.87	−3.41	−0.85	−2.14	−0.54
1 (+4)	39.84	39.30	37.42	36.22	36.30	37.62	−0.54	−0.13	−2.42	−0.61	−3.63	−0.91	−3.55	−0.89	−2.22	−0.56
Average							−0.47	−0.12	−2.25	−0.56	−3.42	−0.86	−3.36	−0.84	−2.25	−0.56

[a] Peptide sequences shown in Table 1.
[b] RP-HPLC conditions, see Section 2.2.
[c] $\Delta t = t_R$ of peptide at a particular salt concentration minus t_R in the absence of salt.
[d] N = number of positively charged residues in peptide.

tide retention times with increasing NaCl concentration of the six peptides relative to those obtained in its absence. As noted previously, the Cl^- ion is considerably more hydrophilic than TFA^-, offering an explanation for this observed behaviour. Thus, with the increasing addition of NaCl to the 10 mM TFA mobile phase, with subsequent increasing displacement of hydrophobic TFA^- anions with Cl^-, the overall effective hydrophobicity of the peptides will be reduced and, hence, retention times will decrease. Note that, in the 10 mM H_3PO_4 system, the Cl^- ion was displacing the even more hydrophilic phosphate ion; hence the immediate rise in retention time with NaCl addition. In the case of $NaClO_4$ and NaTFA, increasing ClO_4^- or TFA^- concentration is enhancing the overall hydrophobicity of the peptides relative to their behaviour in the absence of salt, albeit to a lesser degree than seen previously for the 10 mM H_3PO_4 results (Fig. 1, left panel), since the effective relative hydrophobicities of the peptides in the starting 10 mM TFA mobile phase are already significantly greater than those observed in the starting 10 mM H_3PO_4 system.

3.3. Effect of salts on RP-HPLC retention behaviour of peptides with varying net positive charge

Tables 2–4 (bottom panels) present retention time data obtained from the effect on retention times of peptides 1, 7, 8 and 9 (+4, +3, +2 and +1 net charge, respectively) on the addition of $NaClO_4$ (Table 2), NaTFA (Table 3) or NaCl (Table 4) over a concentration range of 5–100 mM to a mobile phase containing 10 mM H_3PO_4. From Fig. 2, there is again an increase in peptide retention, over that obtained in the absence of salt with increasing salt concentration, in a similar manner to the

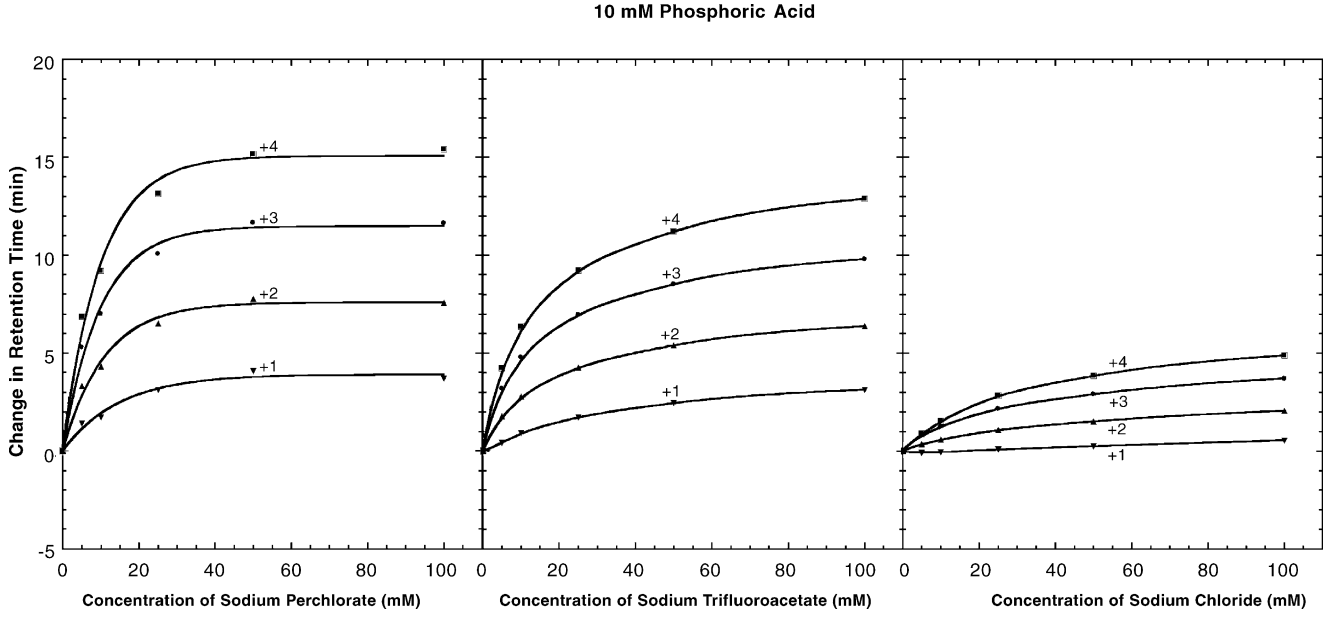

Fig. 2. Effect of salt concentration on RP-HPLC of four model peptides with varying net positive charge (+1, +2, +3, +4) relative to that obtained in the absence of salt. Conditions: same as Fig. 2 for the 10 mM H_3PO_4 system. The sequences of the peptides (peptides 1, 7, 8 and 9) are shown in Table 1.

results observed in Fig. 1 for peptides 1–6. However, it is clear that, for a given salt, the magnitude of this increase is dependent upon the number of positive charges (lysine residues) the peptide contains. Thus, for all three salts, the magnitude of the change in peptide retention time with increasing salt concentration relative to the absence of salt is in the order peptide 9 (+1 net charge) < peptide 8 (+2) < peptide 7 (+3) < peptide 1 (+4). Again, when one assumes an ion-pairing effect, this effect should be greater the more positively charged residues are available to interact with the salt anions, as observed. Support for such an ion-pairing mechanism for the observed results, rather than a simple induction of hydrophobic interactions with the reversed-phase packing with increasing salt concentration, also lies with the above order of the magnitude of changes in peptide retention time with increasing salt concentrations: peptide 9 < 8 < 7 < 1, i.e., in order of decreasing peptide hydrophobicity (Tables 2–5). If an induction of the hydrophobic effect was responsible for the observed results, rather than ion-pairing effects, the reverse order may have been expected, i.e., with increasing salt concentration having the greatest effect on the most hydrophobic peptide (peptide 9) and the least effect on the most hydrophilic peptide (peptide 1).

From Fig. 2, the relative efficacy of the three salts in increasing peptide retention time is again clearly in the order NaCl ≪ NaTFA < $NaClO_4$ for each of the four peptides in the mixture. Indeed, the essentially negligible effect of increasing NaCl concentration on the retention behaviour of peptide 9 (+1), the most hydrophobic of the four peptides, also supports an ion-pairing mechanism for the observed results rather than hydrophobic induction with increasing salt concentration. Fig. 3 presents a visualization of the result of relative effectiveness of the three salts in enhancing interaction of peptides 1, 7, 8 and 9 with the reversed-phase column. The elution profiles shown were obtained in the absence of salt (bottom panel) or in the presence of the same concentration (25 mM) of NaCl, NaTFA and $NaClO_4$. Note that, although all four peptides exhibit an increase in retention time in the presence of salt, since there is a disproportionate effect on peptide retention time, depending on the net positive charge on the peptide (Fig. 2) (peptide 9 < 8 < 7 < 1; +1, +2, +3, +4, respectively) as well as the effectiveness of the salts in enhancing peptide hydrophobicity (NaCl ≪ NaTFA < $NaClO_4$). This results in a decrease in relative separation of the four peptides in the order of 0 mM salt > NaCl ≫ NaTFA > $NaClO_4$.

Finally, Fig. 4 compares the change in retention times of the four peptides relative to those obtained in 10 mM H_3PO_4 per net positive charge on the peptides (Δt/charge). For $NaClO_4$, the convergence of the plots for the four peptides again suggests that the addition of this salt has an essentially identical effect on each positively charged residue in the peptides. A similar, albeit not so clear cut, effect can be seen for NaTFA and NaCl. Overall, it would appear that when increasing the concentration of these two salts, the higher the net charge on the peptide, the greater the effect per charge. Thus,

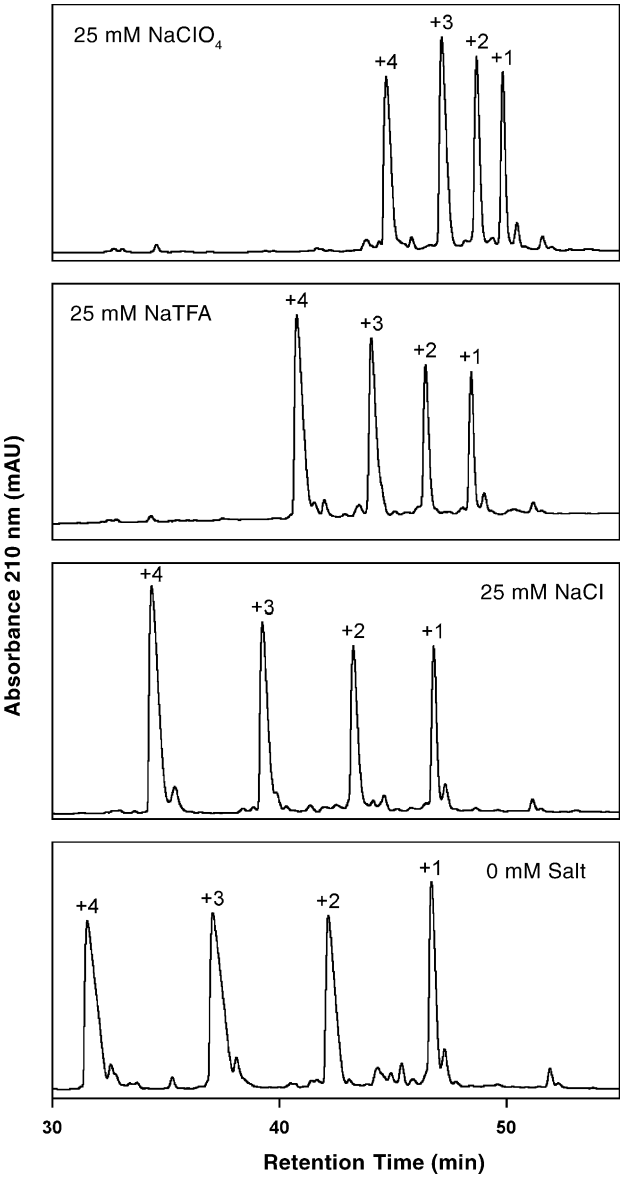

Fig. 3. Effect of salt on RP-HPLC elution profile of model peptides with varying net positive charge (+1, +2, +3, +4). Conditions: linear AB gradient (0.5% acetonitrile/min) at a flow-rate of 0.3 ml/min, where eluent A is 10 mM aq. H_3PO_4 and eluent B is 10 mM H_3PO_4 in 50% aq. acetonitrile, both eluents containing 0 mM salt or 25 mM NaCl, NaTFA or $NaClO_4$; runs were carried out at 25 °C. The sequences of the peptides (peptides 1, 7, 8 and 9) are shown in Table 1.

peptides 1 and 7 (+4 and +3, respectively) show an essentially identical response per net charge with both increasing NaTFA and NaCl concentration; in contrast, peptide 8 (+2) and peptide 9 (+1) exhibit a somewhat lesser response. Note that such results, particularly those for $NaClO_4$, suggest that the response of positively charged peptides to salt concentration variations may be predictable, with potentially useful repercussions for development and optimization of peptide separation protocols.

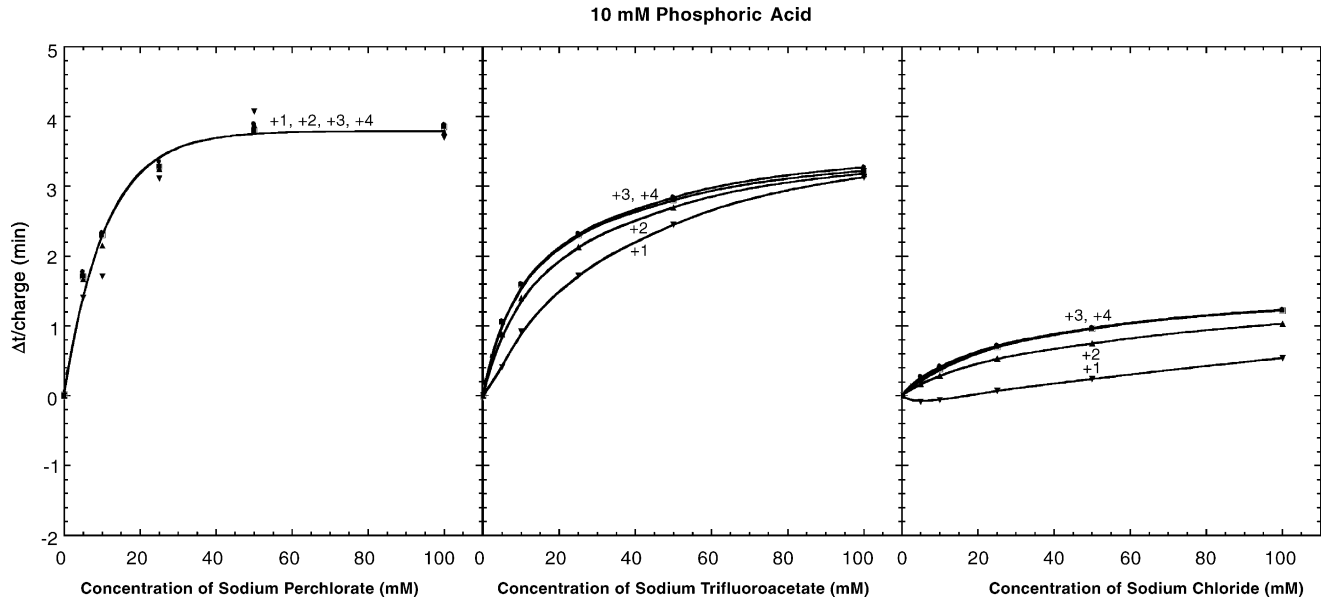

Fig. 4. Effect of salt concentration on the change in RP-HPLC retention time (Δt) per net positive charge relative to that obtained in the absence of salt. Conditions: same as Fig. 2 for the 10 mM H_3PO_4 system. The sequences of the peptides (peptides 1, 7, 8 and 9) are shown in Table 1.

4. Conclusions

The present study investigated the effect of the addition of 5–100 mM NaCl, NaTFA or $NaClO_4$ to acidic RP-HPLC mobile phases on the retention behaviour of two mixtures of synthetic 18-residue peptides, containing either six peptides with the same net positive charge (+4) or four peptides with net charges of +1, +2, +3 and +4. The ClO_4^- anion proved to be a significantly more effective ion-pairing reagent than either the hydrophobic TFA-anion or the hydrophilic Cl^- anion. In addition, such effects were more marked when salt addition was applied to eluents containing 10 mM H_3PO_4 compared to 10 mM TFA. The predictable nature of the effect of increasing perchlorate concentration on the retention behaviour of positively charged peptides also offers useful potential for the development of peptide separation protocols.

Acknowledgement

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

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