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Journal of Chromatography A, 1073 (2005) 137–145
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Comparison of an organic polymeric column and a silica-based reversed-phase for the analysis of basic peptides by high-performance liquid chromatography

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Available online 5 November 2004

Abstract

The performance of a purely polymeric and a Type B silica-based C_{18} reversed-phase column was compared for the analysis of the basic peptide bradykinin and some analogues in order to assess the contribution of silanol interactions to peak shape. Good peak shapes were obtained for small masses of these peptides (0.1 μg or less) using acidic mobile phases on both columns; however, both showed a similar and serious deterioration in peak shape with increasing sample mass. Loss of efficiency on both columns as sample mass increased was considerably more serious when using formic acid rather than trifluoroacetic acid (TFA) as a mobile phase additive. For example, the peak capacity for a 2.5 μg load of one bradykinin on the polymeric column was reduced to only 0.38 times its value for 0.1 μg when using 0.02 M formic acid, compared with 0.77 times its value when using the same concentration of TFA. This result can be attributed to the ion pair effect of TFA and its higher ionic strength, which reduce mutual repulsion of charged peptides when held on the hydrophobic surface of the phase. Addition of salt (KCl) to the formic acid mobile phase caused dramatic increases in retention on the polymeric column, which can also be attributed to ion-pairing effects between halide ions and peptides. The increase in retention with salt addition also confirms that there are no ionic retention sites on the polymeric phase at low pH. The general similarity in behaviour between the polymeric and silica column suggests that silanol groups have little involvement in the retention and overload behaviour of these peptides when using highly inert Type B silica phases.

Keywords: Basic peptides; Silanol interactions; Overloading

1. Introduction

The analysis of complex peptide mixtures using high-performance liquid chromatography (HPLC) in combination with MS is a most important methodology in the identification of proteins in proteomic studies. Individual peptides separated during the HPLC stage can be identified by tandem MS techniques, which can automatically perform partial amino acid sequencing for comparison with library data [1]. Peptide mixtures can be extremely complex, and their separation is therefore best performed using high efficiency columns. The peak shapes of basic peptides can be affected by silanol interactions which have been demonstrated on older Type A (impure) silica-based ODS phases even at low pH [2].

In a previous publication, we studied the chromatography of a mixture of synthetic model basic peptides on a Type B pure silica-ODS phase [3]. This study seemed to indicate that overload of the column, caused by mutual repulsion of ions held on the hydrophobic C_{18} phase, rather than silanol interaction, could be the major cause of tailing and peak shape problems with basic peptides at low pH. Furthermore, overload seemed to be much worse in formic acid than trifluoroacetic acid (TFA), despite the fact that formic acid is often preferred because it gives rise to less signal suppression in MS work. The differences in these additives were attributed to differences in their ionic strength and ion-pair capability. However, it was not proved unequivocally that silanol groups were not re-

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doi:10.1016/j.chroma.2004.10.023

sponsible for poor peak shapes of basic peptides at low pH.

In the present study, we aimed to compare the performance of a purely polymeric column with that of a pure silica-ODS column, using both formic acid and TFA as additives. As the polymer column has no silanol groups, it allows a useful comparison with a silica-based phase, enabling the influence of silanol groups on performance for small and overloaded samples to be observed. Traditionally, purely polymeric columns suffer from rather low efficiency compared with silica-based phases [4]; however, 3 μm particle size columns are now available, giving improved efficiency (albeit at the expense of higher operating pressure). A secondary aim of the study was to evaluate the performance of these newer columns for peptide analysis. Polymeric phases, which are often based on a polystyrene–divinylbenzene matrix, have the advantage of pH stability over silica-based phases. They conceivably may give less stationary phase bleed in acidic mobile phase as they have no bonded ligands to hydrolyse at acid pH, which may be an advantage in HPLC–MS studies. In the present study, we used the naturally occurring basic peptide bradykinin (two basic arginine residues) and related bradykinin fragments/compounds with one and three arginine residues. These peptides are commercially available in high purity, with certified peptide content established by the supplier using amino acid analysis, enabling accurate preparation of standards. The Alberta peptide mixture used previously [3] is in contrast supplied as a qualitative test, with the individual compounds not commercially available. It contains model synthetic peptides with one to four basic lysine residues where the carboxy terminals are amidated and the N-terminals acetylated. Thus, the charge on the peptides results only from the charge on the side chains and not on the N or C terminals of the peptides. Conceivably, these four model peptides could behave differently from normal peptide fragments. The charge on the bradykinins arises in contrast from charges on the side chains and on the N-terminal groups. The use of a completely different set of related peptides to the Alberta mixture allows comparison with putative trends suggested by the previous study [3]. Finally, we wished to study the effect of gradient steepness on overload. It seems possible that overload in gradient elution might be influenced by the gradient retention factor k^*, in the same way that overloading in isocratic separations is influenced by the retention factor k. k^*, the average retention factor in gradient elution is a function of gradient steepness as shown by the equation:

$$k^* = \frac{87 t_g F}{(\Delta\%B) V_m S} \quad (1)$$

where F is the flow rate, $\Delta\%B$ the gradient range expressed as the change in volume fraction of B, V_m the column void volume and t_g is the gradient time [5]. S is obtained from the variation of isocratic retention factor with solvent composition, where:

$$\log k = \log k_w - S\phi \quad (2)$$

k_w is the retention of the solute in pure water, ϕ the volume fraction of organic modifier and S a factor depending on the solute and the modifier [5]. Thus, although shallow gradients should increase the peak capacity of a system [6], it is possible that they might lead to greater problems with overloading, as indicated by theoretical considerations of peak overload [2,5].

2. Experimental

An 1100 binary high pressure mixing gradient HPLC system (Agilent, Waldbronn, Germany) with Chemstation, UV detector (1 μl flow cell), and Rheodyne 7725 valve (5 μl injections) was used in all experiments. Connections were made with minimum lengths of 0.01 cm i.d. tubing to minimise extra-column volume. Temperature was maintained at 30 °C by immersing the column and injector in a thermostatted water bath model W14 (Grant Instruments, Cambridge UK). A 3 m × 0.5 mm i.d. length of stainless steel tubing connected between the pump and injector and also immersed in the bath was used to preheat the mobile phase; flow was 1.0 $cm^3\ min^{-1}$. Gradient retention times were not corrected for the small gradient delay produced, which remained constant in all experiments. The columns used were Discovery C_{18}, 5 μm particle size, pore diameter 19 nm, surface area 194 $m^2\ g^{-1}$ (for unmodified silica) 25 cm × 0.46 cm i.d. (Supelco, Bellafonte, USA) and PLRP-S 3 μm particle size, pore diameter 10 nm, 15 cm × 0.46 cm i.d. (Polymer Laboratories, Church Stretton, UK). Peak widths at half height were determined using the Chemstation. The asymmetry factor (A_s) was calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak. Column void volume was measured by injection of uracil. Buffer additives were incorporated in both "A" and "B" solvents in the gradient to maintain a constant concentration throughout the gradient. Ionic strength calculations were performed using the PHoEBuS program (Analis, Orleans, France) using correction of activity coefficients according to the Debye–Hückel equation.

Bradykinin, bradykinin fragment 1-8, bradykinin fragment 2-9 and Arg-[Hyp3, Phe 7] bradykinin were obtained from Sigma–Aldrich (Poole, UK). The peptide content of these substances, determined by amino acid analysis, was available from Sigma–Aldrich.

3. Results and discussion

Bradykinin is a basic peptide that has important biological functions which include the regulation of fluid and electrolyte balance, vasodilation and capillary permeability. Its amino acid structure, together with the structure of related peptides used in this study, is shown in Table 1. These bradykinins contain from 1 to 3 basic arginine amino acid residues. The pK_a of arginine residues within peptide chains is above 12,

Table 1
Amino acid composition of bradykinin and related peptides

Peptide	Amino acid sequence	Charge (pH 2.7)
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	+3
Bradykinin fragment 1-8	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	+2
Bradykinin fragment 2-9	Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	+2
Arg-[hydroxypro^3, Phe^7] bradykinin	Arg-Arg-Pro-hydroxyPro-Pro-Gly-Phe-Ser- Phe-Phe-Arg	+4

Arg: arginine; Gly: glycine; hydroxyPro: hydroxyproline; Pro: proline; Phe: phenylalanine; Ser: serine.

thus the basic side chains are completely ionised along with the N-terminal group under the conditions of our study (pH 2.7 or less). Conversely, at pH 2.7 or less, C-terminal carboxyl groups are little ionised, having pK_a values in polypeptides typically 3.6 or above [7]. Therefore, the bradykinins show net positive charges of 2–4 under the conditions of our study (see Table 1).

3.1. Comparison of loadability of polymeric and silica column using formic acid and TFA mobile phases

Table 2 shows retention times, peak widths at half height, asymmetry factor and peak capacity for the four compounds using 0.09% formic acid (0.02 M) and 0.09% TFA (0.0079 M) with the polymeric column, and Table 3 gives similar results for the silica-ODS phase. Peak capacity was measured using the equation:

$$P = 1 + \frac{t_g}{1.699 w_{0.5}} \quad (3)$$

where $w_{0.5}$ is the peak width at half height and t_g the gradient time. t_g was calculated for a 5–42.5% acetonitrile gradient. Thus, t_g is 30 min for a gradient of 1.25% acetonitrile min^{-1} (60 min for 0.625% ACN min^{-1}, 15 min for 2.5% ACN min^{-1}). For tailing or overloaded peaks this equation may give an optimistic value of the peak capacity as noted previously [3]. We studied chromatographic behaviour in some detail with these additive concentrations, because they are typically employed in HPLC–MS analysis. Higher molar concentrations of TFA are likely to give considerable MS suppression effects [8]. However, a brief comparison of performance with equimolar concentration of TFA (0.02 M) is detailed below. Tables 2 and 3 show that for all column/mobile phase combinations, rather minor improvements in peak shape are obtained when reducing the injected sample concentration from 50 to 20 mg l^{-1} (0.25–0.1 μg sample mass). Reducing sample mass below 0.1 μg gave rise to increased noise and imprecision in the measurements. Thus, for practical purposes, results with 20 mg l^{-1} solutions (0.1 μg injected on-column, "low load") were taken as giving optimum peak shape. (*Note*: having established this limit using a medium gradient, injections of 0.25 μg of peptide were not carried out for slow and fast gradients). Also shown in Tables 2 and 3 is the fractional peak capacity, which is the peak capacity for a 500 mg l^{-1} injection (2.5 μg on-column, "high load") of the analyte divided by that for 20 mg l^{-1} injection (0.1 μg, "low load") using the same mobile phase conditions. This ratio gives an idea of the loss of resolution which might be obtained due to overloading of the column. Note that 2.5 μg on-column would not at all be a sample mass considered likely to overload a column of the dimensions used with a neutral solute [2].

Tables 2 and 3 indicate a loss of peak capacity on both columns when using "high load" compared with a low load, which is considerable when using formic acid. Considering a medium gradient slope (1.25 % acetonitrile min^{-1}), the fractional peak capacity on Discovery C_{18} with formic acid is 0.66–0.83 for the four bradykinins (Table 3), whereas on the polymer column the results are worse, ranging from 0.38 to 0.63 (Table 2). Thus, the peak capacity for the high load of Arg-bradykinin on the polymer column with formic acid is barely one-third of the value for the dilute solution. Clearly, the results would be much worse for sample masses in excess of 2.5 μg. For the same gradient slope with TFA instead of formic acid, the corresponding range of fractional peak capacity is 0.86–0.93 for Discovery C_{18} and 0.64–0.84 for the PLRP-S, indicating considerably less detrimental effect of sample load when using TFA. Overlaid chromatograms of high and low sample load for formic acid and TFA are shown in Fig. 1 for PLRP-S and Fig. 2 for Discovery C_{18}. Peaks for high load in formic acid on both columns tend towards right-angled triangle shapes characteristic of overloading. Retention times decrease with increased sample mass (Figs. 1 and 2) which is again characteristic of overloading [5]. Kinetic effects such as silanol interactions give rise instead to exponential tailing which is mostly not observed in these chromatograms. Similar peak shapes are shown with TFA, but clearly the extent of overload is much less than with formic acid, despite the lower molar concentration of TFA used (0.0079 M TFA compared with 0.02 M formic acid).

Comparing the peak capacities for "low load" using formic acid or TFA on either column shows relatively small differences. For example, on PLRP-S using a medium gradient slope, Arg-bradykinin gave a peak capacity of 183 and 190 using formic acid and TFA respectively; on Discovery C_{18}, the comparable values for the same peptide were 159 and 190. In contrast, for high load of this peptide, the peak capacities on PLRP-S were 69 and 121 with formic acid and TFA, respectively and on Discovery C_{18}, 105 and 163, respectively. These results indicate that as long as the load is small, the differences between formic acid and TFA are relatively small but increase as the sample load increases.

Table 2
Comparison of peak shapes using different buffers, PLRP-S

Peptide	Sample mass (μg)	t_r (min)	$w_{0.5}$	A_s	Peak capacity	Fractional peak capacity
0.02 M formic acid pH 2.7						
Slow gradient 0.625% ACN min^{-1}						
Bradykinin	2.5	22.0	0.413	4.5	87	0.56
	0.1	22.5	0.229	2.2	155	
Fragment 1-8	2.5	27.7	0.573	4.0	63	0.66
	0.1	28.2	0.375	3.1	95	
Fragment 2-9	2.5	25.8	0.451	4.8	79	0.56
	0.1	26.3	0.254	2.1	140	
Arg-Brady	2.5	26.7	0.461	6.0	78	0.38
	0.1	27.2	0.174	3.0	205	
Medium gradient 1.25% ACN min^{-1}						
Bradykinin	2.5	14.1	0.245	5.5	73	0.56
	0.25	14.4	0.144	2.7	124	
	0.1	14.4	0.137	2.5	130	
Fragment 1-8	2.5	17.3	0.363	4.4	50	0.63
	0.25	17.6	0.237	3.0	76	
	0.1	17.5	0.226	2.9	79	
Fragment 2-9	2.5	16.3	0.265	5.3	68	0.60
	0.25	16.4	0.166	2.9	107	
	0.1	16.5	0.157	2.9	114	
Arg-Brady	2.5	16.5	0.261	6.8	69	0.38
	0.25	16.9	0.115	3.0	155	
	0.1	16.8	0.097	2.3	183	
Fast gradient 2.5% ACN min^{-1}						
Bradykinin	2.5	9.6	0.137	4.9	65	0.58
	0.1	9.8	0.079	2.3	113	
Fragment 1-8	2.5	11.3	0.184	5.4	49	0.65
	0.1	11.4	0.119	3.0	75	
Fragment 2-9	2.5	10.8	0.154	5.4	58	0.59
	0.1	10.9	0.091	2.9	98	
Arg-Brady	2.5	10.7	0.135	5.0	67	0.44
	0.1	10.9	0.058	2.1	153	
0.0079 M TFA pH 2.3						
Slow gradient 1.25% ACN min^{-1}						
Bradykinin	2.5	29.5	0.257	2.8	138	0.78
	0.1	29.7	0.201	1.5	176	
Fragment 1-8	2.5	33.7	0.354	2.6	101	0.81
	0.1	34.1	0.288	1.7	124	
Fragment 2-9	2.5	31.5	0.339	2.8	105	0.81
	0.1	31.6	0.273	2.0	130	
Arg-Brady	2.5	35.5	0.244	3.5	146	0.64
	0.1	35.8	0.157	1.4	227	
Medium gradient 1.25% ACN min^{-1}						
Bradykinin	2.5	18.2	0.156	2.6	114	0.78
	0.25	18.3	0.123	1.6	145	
	0.1	18.3	0.122	1.5	146	
Fragment 1-8	2.5	20.6	0.210	2.6	85	0.84
	0.25	20.7	0.179	2.0	100	
	0.1	20.7	0.177	1.9	101	
Fragment 2-9	2.5	19.3	0.203	3.2	88	0.82
	0.1	19.3	0.167	2.5	107	
Arg-Brady	2.5	21.1	0.147	2.7	121	0.64
	0.25	21.3	0.098	1.5	182	
	0.1	21.3	0.093	1.3	190	
Fast gradient 2.5% ACN min^{-1}						
Bradykinin	2.5	11.7	0.091	2.6	98	0.83
	0.1	11.8	0.075	1.6	118	
Fragment 1-8	2.5	13.0	0.125	2.8	72	0.86
	0.1	13.1	0.107	2.1	84	

Table 2 *(Continued)*

Peptide	Sample mass (μg)	t_r (min)	$w_{0.5}$	A_s	Peak capacity	Fractional peak capacity
Fragment 2-9	2.5	12.4	0.121	3.6	74	0.82
	0.1	12.4	0.100	2.5	90	
Arg-Brady	2.5	13.2	0.087	2.8	102	0.68
	0.1	13.3	0.060	1.3	149	

Clearly, the 3 μm polymeric column gives satisfactory results for the analysis of these strongly basic peptides, broadly comparable with those obtained on the 5 μm silica ODS phase. A quantitative comparison of peak capacity and overload on the polymeric and silica column is difficult: the columns have different dimensions, different particle size and are based on different materials. Furthermore, we did not scale the gradient according to Eq. (1), to take into account the different column lengths and solute *S* values, so that the same value of k^* would be obtained on both columns. However, it

Table 3
Comparison of peak shapes using different buffers, Discovery C_{18}

Peptide	Sample mass (μg)	t_r (min)	$w_{0.5}$	A_s	Peak capacity	Fractional peak capacity
0.02 M formic acid pH 2.7						
Slow gradient 0.625% ACN min^{-1}						
Bradykinin	2.5	22.2	0.343	4.1	104	0.69
	0.1	22.7	0.237	2.4	150	
Fragment 1-8	2.5	28.4	0.416	2.9	86	0.83
	0.1	28.8	0.342	2.1	104	
Fragment 2-9	2.5	26.3	0.334	3.9	107	0.65
	0.1	26.7	0.215	2.0	165	
Arg-Brady	2.5	27.3	0.341	7.1	105	0.54
	0.1	28.0	0.183	3.3	194	
Medium gradient 1.25% ACN min^{-1}						
Bradykinin	2.5	14.6	0.184	3.6	97	0.78
	0.25	14.7	0.142	2.5	125	
	0.1	14.7	0.142	2.4	125	
Fragment 1-8	2.5	18.0	0.243	2.9	74	0.83
	0.25	18.1	0.203	2.3	88	
	0.1	18.1	0.200	2.2	89	
Fragment 2-9	2.5	17.0	0.191	3.8	93	0.71
	0.1	17.2	0.136	2.3	131	
Arg-Brady	2.5	17.1	0.170	4.6	105	0.66
	0.25	17.3	0.113	3.4	157	
	0.1	17.3	0.112	3.4	159	
Fast gradient 2.5% ACN min^{-1}						
Bradykinin	2.5	10.3	0.104	3.4	86	0.80
	0.1	10.4	0.083	2.3	108	
Fragment 1-8	2.5	12.1	0.143	3.5	63	0.83
	0.1	12.2	0.118	2.3	76	
Fragment 2-9	2.5	11.6	0.113	3.8	79	0.75
	0.1	11.7	0.084	2.5	106	
Arg-Brady	2.5	11.5	0.091	3.5	98	0.76
	0.1	11.6	0.069	2.7	129	
0.0079 M TFA pH 2.3						
Medium gradient 1.25% ACN min^{-1}						
Bradykinin	2.5	19.7	0.121	1.7	147	0.92
	0.25	19.7	0.111	1.4	160	
	0.1	19.8	0.111	1.4	160	
Fragment 1-8	2.5	22.3	0.175	1.9	102	0.93
	0.25	22.5	0.163	1.5	109	
	0.1	22.4	0.162	1.5	110	
Fragment 2-9	2.5	20.8	0.163	2.2	109	0.88
	0.25	20.8	0.144	1.8	124	
	0.1	20.8	0.143	1.7	124	
Arg-Brady	2.5	23.2	0.109	1.8	163	0.86
	0.25	23.4	0.095	1.1	188	
	0.1	23.3	0.093	1.2	190	

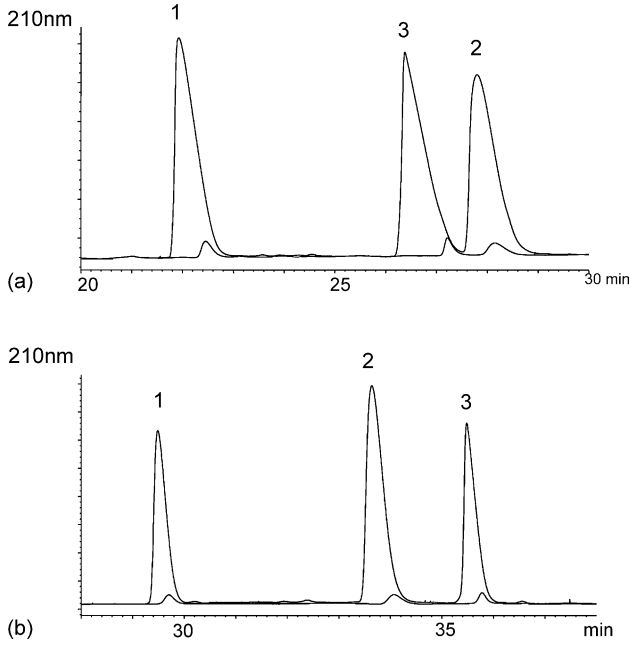

Fig. 1. Analysis of bradykinins on PLRP-S. Gradient 0.625% ACN min^{-1}. Flow rate: 1 ml min^{-1}; detection: UV at 210 nm. Peak identities: (1) bradykinin; (2) bradykinin fragment 1-8; (3) 3-Arg bradykinin. (a) Mobile phase additive formic acid (overall 0.02 M). (b) Mobile phase additive TFA (overall 0.079 M).

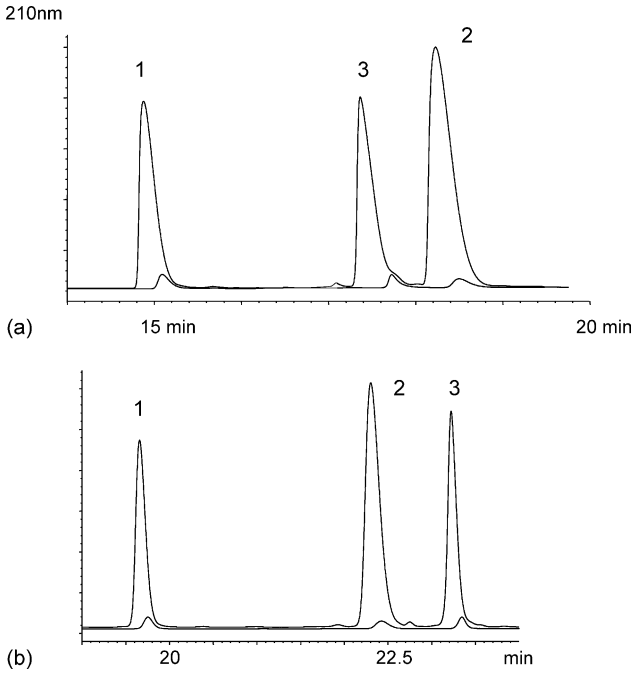

Fig. 2. Analysis of bradykinins on Discovery C_{18}. Conditions as Fig. 1 except gradient 1.25% ACN min^{-1}. (a) Mobile phase additive formic acid (overall 0.02 M). (b) Mobile phase additive TFA (overall 0.0079 M).

does seem that both columns overload in a broadly similar fashion. This result suggests that the overload mechanism involves the hydrophobic portion of the stationary phase in the silica column, rather than silanol groups, since the polymer phase possesses no silanol groups. The finding lends weight to our hypothesis that mutual repulsion of ions held on the hydrophobic portion of the phase largely causes overload of samples [4,9]. However, some degree of silanol interaction of these highly charged basic peptides with a few highly acidic groups on the silica-based stationary phases cannot be entirely discounted. The degree of this interaction is likely to vary according to the particular type of silica phase employed.

Previously, with the Alberta mixture, where peptides had very similar gradient peak width, it was noted that loadability decreased in line with increasing peptide charge. This result is not contradicted by the present study, with the worst fractional peak capacities obtained for the most highly charged peptide Arg-bradykinin, and the best for the doubly-charged bradykinin fragments on both columns under all conditions. However, it is not possible to draw firm conclusions from the present data due to the differences in peak width shown for the different bradykinins under non-overloaded conditions (see Tables 2 and 3).

3.2. Effect of TFA concentration on loadability of the phase

Results in Tables 2 and 3 were obtained using 0.0079 M TFA. 0.02 M TFA is a higher concentration than generally used in HPLC–MS studies due to the suppression effects of this acid on the detector signal. However, we briefly investigated use of 0.02 M TFA such that comparison of loadability with equimolar amounts of each acid could be made. 0.02 M TFA has a pH below 2, so its aqueous solution was adjusted to pH 2.7 with concentrated ammonia solution, for comparison with the formic acid results. Because TFA is a relatively strong acid, addition of ammonia hardly changes the ionic strength from that of acid alone; 0.02 M TFA has ionic strength = 19 mM, whereas 0.02 M TFA adjusted to pH 2.7 with concentrated ammonia solution has ionic strength = 20 mM. The same is not true for weaker acids such as formic acid, in which case the ammonium salt of the acid is dissociated whereas the acid is only weakly ionised [3]. Table 4 shows that the column capacity increases using 0.02 M compared with 0.0079 M TFA (note: fragment 1-8 was not included in this study as it gave similar results to fragment 2-9 using the lower concentration of TFA). The fractional peak capacity for the bradykinins in Table 4 ranges from 0.77 to 0.95 in 0.02 M TFA compared with 0.64–0.84 in 0.0079 M TFA. Thus, comparing equimolar solutions of the additive at the same pH, TFA is seen to be much more effective still in reducing overload problems than formic acid (recall 0.02 M formic acid gave peak capacities 0.37–0.64). It is possible that the higher ionic strength of TFA contributes to lessening of mutual repulsion effects between protonated peptides held on the surface of the phase. However, ion pair

Table 4
Analysis of bradykinins on PLRP-S with TFA concentration 0.02 M adjusted to pH 2.7 with ammonia

	Sample mass (μg)	t_r (min)	Fractional peak capacity
Medium gradient 1.25% ACN min^{-1}			
Bradykinin	2.5	19.0	0.91
	0.1	19.1	
Fragment 2-9	2.5	19.6	0.95
	0.1	19.7	
Arg-Brady	2.5	22.2	0.77
	0.1	22.4	

effects may be a major contributor to differences in loadability shown when different mobile phase additives are used [10]. If some of the peptide is held as (neutral) ion pairs with TFA on the surface of the phase, reduced mutual repulsion and an increase in loadability will result. The ion pair effects of TFA can be seen in increased retention of all the peptides even when using 0.0079 M TFA compared with 0.02 M formic acid (Table 2). Further increases in retention are shown as the concentration of TFA is increased to 0.02 M (Table 4), consistent with the ion-pair effect of TFA. Note that a rather similar argument holds if "dynamic ion interaction" rather than a classical ion pair effect occurs.

3.3. Effect of salt and its concentration on peptide retention and peak shape

It has been assumed so far that PLRP-S behaves as a pure hydrophobic surface at low pH with no ionic retention sites. For similar polymeric columns at low pH, we established this to be true; however, negatively charged sites causing ionic retention of basic drugs were demonstrated on some polymeric columns at neutral pH [4]. These sites can be the cause of tailing on purely polymeric columns. We investigated the effect of addition of salt (KCl) on retention in formic acid buffer using the PLRP-S column since this particular phase was not studied in the previous investigation. If negatively-charged retention sites exist, then competitive interactions with salt cations should reduce retention as indicated by Cox and Stout [11], and later in detailed studies by Carr and coworkers [12,13]. Table 5 shows the effect on retention of the peptides of adding increasing concentrations of KCl to the mobile phase in isocratic analysis using formic acid as the buffer. It can be seen that very substantial increases in retention time, together with improvement in peak shape, are obtained as the concentration of salt is increased over the range 0.005–0.02 M, with a particularly large difference between 0.005 M and no KCl added. The large increases in retention time with added salt are surprising although can also be observed in our previous results for the Alberta peptides when using gradient elution [3]. Gradient elution rather conceals the effect of added salt, because change in organic solvent composition has such a powerful effect on peptide retention due to high S values (see below). The retention increase could also be attributed to electrostatic interaction between the protonated peptide and added chloride anion, forming some sort of ion-association or "ion pair " complex in the stationary or mobile phase. This complex is expected to have greater hydrophobicity and thus increased retention compared with the hydrated peptide cation [14–17]. Despite improved column efficiency on addition of salt, Table 5 indicates generally low efficiency for these peptides in comparison with non-ionogenic compounds which typically give around 10,000 plates on this column. It was shown previously that the effect of overloading was much more serious in isocratic rather than gradient elution separations [3], and indeed peaks appeared visibly overloaded even at this relatively low injected sample mass (0.25 μg), even in the presence of added salts. We did not reduce the sample mass further due to noise and its effect on the reproducibility of measurements. The improvement in column efficiency on addition of salt can again be attributed to reduced overloading of the phase brought about by decreased mutual repulsion of adsorbed peptides ions, caused by ion pairing with chloride and/or the increased ionic strength of the mobile phase.

Table 5
Comparison of performance for PLRP-S on addition of KCl

	Sample mass (μg)	KCl (M)	t_r (min)	N	A_s
Isocratic analysis, overall 0.02 M formic acid, 17% ACN					
Bradykinin	0.25	0.00	4.4	1650	3.6
		0.005	7.1	2350	2.2
		0.01	8.2	2630	1.9
		0.02	9.5	2750	1.8
Arg-Brady	0.25	0.00	11.0	1750	4.5
		0.005	23.2	3630	3.1
		0.01	29.0	4451	2.8
		0.02	36.6	4870	1.8

N is the number of theoretical plates in the column.

In an attempt to investigate further the extent of the contribution of these individual effects, we also added 0.01 M KF or 0.01 M KBr instead of 0.01 M KCl to the formic acid mobile phase of Table 5, giving the results in Table 6. Anions with small ionic radius are less polarisable with limited charge delocalisation, and should give rise to smaller ion pair effects [18]. Thus, ion pair effects might be expected to increase in accord with the ionic radius of fluoride, chloride and bromide (119×10^{-12}, 167×10^{-12} and 182×10^{-12} m, respectively). The experimental results (Tables 5 and 6) suggest that addition of any of these salts produces a large increase

Table 6
Comparison of performance for PLRP-S on addition of different salts

	Sample mass (μg)	Salt	t_r (min)	N	A_s
Isocratic analysis, overall 0.02 M formic acid, 17% ACN					
Bradykinin	0.25	0.01 M KF	7.9	1500	2.4
		0.01 M KCl	8.2	2630	1.9
		0.01 M KBr	9.8	2480	2
Arg-Brady	0.25	0.01 M KF	28.5	2300	3.5
		0.01 M KCl	29.0	4451	2.8
		0.01 M KBr	37.6	4470	2.6

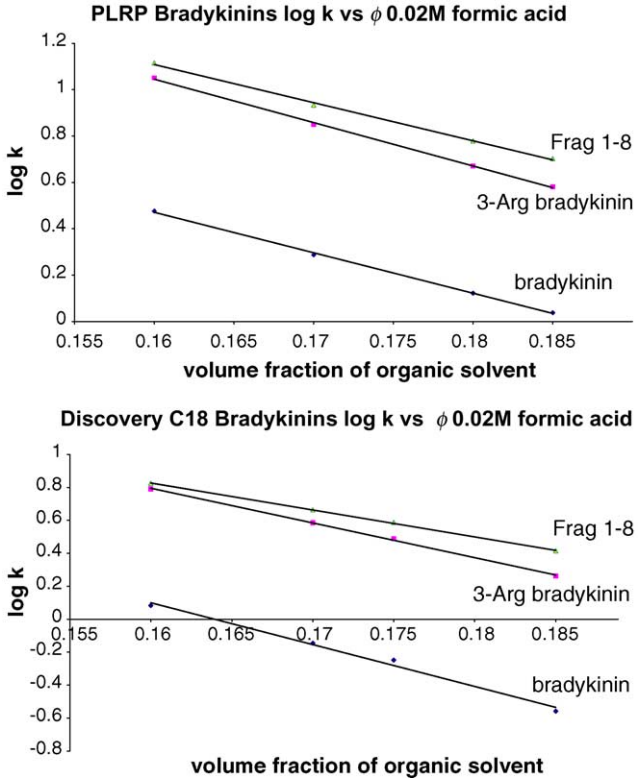

Fig. 3. Plot of log k vs. volume fraction of acetonitrile in the isocratic mobile phase (overall 0.02 M formic acid) for bradykinins and analogues on polymeric and silica ODS phase. For other conditions, see Fig. 1.

in retention compared with formic acid containing no added salt. Additionally, chloride and bromide produce incremental increases in retention compared with fluoride consistent with the likely greater "ion pair" ability of ions of larger ionic radius. Furthermore, peak shape generally improves in line with the increasing "ion pair" ability of the anion.

3.4. Comparison of S values on polymeric and silica-ODS column

Because S values influence the value of k^* in gradient elution, then S may influence column overload. Fig. 3 shows plots of log k versus % organic solvent in the mobile phase for isocratic analysis using formic acid on the polymer and silica ODS column, and Table 7 shows the values of S obtained from the slope of the lines. S values are seen to be rather similar on both columns, which would be expected if the retention mechanism was similar. Thus, it appears that S values are not an important consideration when comparing similarities and differences in overload properties of these two rather different types of column. Values are generally similar to those found for the Alberta peptides on the Discovery C_{18} column [3] and are generally higher than those found for smaller molecules, where S values tend to be ~4 [19].

Table 7
S values for bradykinins on polymeric and silica-ODS phase using formic acid as buffer

Peptide	S (PLRP-S)	S (Discovery C_{18})
Bradykinin	17	25
Bradykinin fragment 1-8	16	16
Arg-Brady	19	21

3.5. Effect of gradient slope on overloading of peptides

We studied finally the effect of gradient slope on overloading of peptides. Table 2 shows the peak capacities using gradient slopes of 0.625 and 2.5% acetonitrile min^{-1} using formic acid for both the polymeric and silica-ODS phases. These slopes represent half and twice the rate of increase discussed so far. Eq. (1) shows k^* is inversely proportional to the gradient slope, thus it might be expected that the effect of overloading would be reduced with fast gradients. For both columns with 0.1 μg peptide where no overloading effects are observed, the peak capacity of the system clearly increases as the gradient slope decreases, in line with theory [6]. For the PLRP-S column, the fractional peak capacity (the ratio of the peak capacity for high sample load divided by peak capacity for small sample load for a given column and mobile phase gradient) is only slightly improved for the fast gradient. For instance, the average fractional peak capacity for the four bradykinins is 0.54 using the slowest gradient compared with 0.57 for the fastest gradient with the PLRP-S column using formic acid. For the C_{18} column with formic acid, the average fractional peak capacity is 0.68 for the slow gradient compared with 0.79 for the fast gradient. These empirical results show a rather small dependence of the degree of overloading on gradient slope. Snyder [5] gives the equation:

$$W_{\text{base}}^2 = \frac{16t_0^2G^2(1+k_\text{f})^2}{N_0} + \frac{6t_0^2k_\text{f}^2w_\text{x}}{w_\text{s}} \quad (4)$$

where W is the peak width at base, N_0 the column efficiency for a small sample mass, k_f the final value of k when a solute band reaches the end of the column (i.e. at the time of elution), w_x the sample mass and w_s is the saturation capacity. G is the gradient compression factor where:

$$G^2 = \frac{(1+p+p^2/3)}{(1+p)^2} \quad (5)$$

and $p = 2.3b$ where b is the gradient steepness factor given by $b = 1/2.3k_\text{f}$. This equation may give only approximate predictions of the bandwidth [5]. However, modelling on a spreadsheet confirms the prediction that for a given value of the column saturation capacity w_s, the degree of overloading will increase as the gradient slope decreases. For example, with a column of $N = 20{,}000$ plates, solute S value ~20 and saturation capacity 1 mg, the equation predicts the ratio of peak widths for a 2.5 μg divided by a 0.1 μg injection as approximately 1.9 for the "fast" gradient, 2.4, for the medium gradient and 2.8 for the "slow" gradient. These can be compared with actual values for Arg-Brady using Discovery C_{18} and formic acid of 1.3, 1.5 and 1.9. Thus, there is at least a rea-

sonable agreement between this equation and experimental results, showing a relatively small effect of gradient slope on overloading over the range of gradients normally employed in peptide separations. Evaluation of the effectiveness of this equation in predicting peak widths would be better carried out with simpler solutes; there is variability in the gradient peak widths of the individual peptides used in our study which can be attributed to the complexity of their structures and stationary phase interactions. In conclusion, it seems that slower gradients may be used to improve the peak capacity of a column without increasing overloading effects seriously for peptides present in higher concentrations.

Similar results are apparent for the PLRP-S column when using TFA, with slight improvement in fractional peak capacity as the gradient slope is increased (Table 2).

4. Conclusions

Purely polymeric columns as well as inert silica-ODS phases can give good results for the analysis of strongly basic peptides at low pH. However, peak shape on both types of column deteriorates with increasing sample load. The similar loading behaviour of both columns suggest that silanol groups are hardly involved in this process, at least when using highly inert ODS phases based on pure silica. Instead, it is more likely that mutual repulsion of charged species held on the hydrophobic portion of the stationary phase is responsible for overload. Overloading effects seem to generally increase as the charge on the peptide is increased. Overloading effects are much more serious when formic acid is used as the mobile phase buffer rather than TFA, especially when equimolar concentrations of the additive are considered. However, lower molar concentrations of TFA (approximately one-third compared with formic acid) are still more effective in reducing overloading compared with formic acid. Overloading does not seem to show a strong dependence on gradient steepness. Reduced overloading in TFA may be caused by the ion pairing ability and higher ionic strength of this acid, which reduce the effect of mutual repulsion of peptide ions held on the hydrophobic surface of the phase. Similar arguments may apply when salt solutions are added to a formic acid mobile phase.

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