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Study of overloading of basic drugs and peptides in reversed-phase high-performance liquid chromatography using pH adjustment of weak acid mobile phases suitable for mass spectrometry

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Abstract

Serious losses in column efficiency for ionised basic drugs and peptides occur due to overloading of C18 phases when weak acid mobile phases of low ionic strength suitable for mass spectrometric detection are used. Measurable changes in retention time and efficiency can be observed even for levels around 0.01 μg of basic drugs on 0.46 cm I.D. columns; the overloading process is a continuum, rather than an event which takes place only once a certain threshold value has been reached. Overloading can be reduced by increasing the mobile phase pH, e.g to the pK_a of the weak acid, which increases the ionic strength, allowing a greater degree of ion pairing and perhaps also physical screening of the adsorbed solute ions by buffer ions. Buffer capacity is also optimum at its pK_a. Silanol ionisation and kinetic tailing effects were mostly absent at aqueous pH up to 4.75 on the hybrid inorganic-organic C18 phase employed in this study, except when analysing the most highly charged peptides. The exact cause of overloading of these ionic species is unclear.
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1. Introduction

The more widespread use of mass spectrometry as a detector for HPLC has increased the use of volatile buffers and mobile phase additives. Mass spectrometric detection is frequently used in the pharmaceutical industry and in forensic science, also for measurement of biomedically important compounds in body fluids, and for peptide analysis in proteomics. Non-volatile mobile phase constituents (e.g. phosphate) lead to decreased signal intensities and signal to noise ratios as well as contamination of the ion source [1]. The analysis of both basic drugs and peptides is commonly carried out at low pH in order to suppress the detrimental interactions which can take place between these protonated species and ionised silanols on the surface of RP columns. For UV detection, phosphate buffers are very commonly employed, typically over the pH range 2.1–3.1 where phosphate (p$K_a \sim 2.1$) gives good buffering while ensuring reasonable long term stability of the bonded phase. In phosphate, peak shape for ionised drugs and peptides deteriorates rather rapidly with increasing sample mass even when using modern highly inert columns [2–4]. Substitution of phosphate with volatile additives such as formic acid and acetic acid can give rise to worse overloading problems. For example, as little as 0.1 μg of the basic drugs nortriptyline and diphenhydramine caused loss of efficiency of up to 30% for a 25 × 0.46 cm column when using 0.02 M formic acid, a commonly used MS compatible mobile phase. Some new phases (e.g. Water Xterra) do not appear to have ionised silanols on their surface at low pH. They give excellent peak shapes for small amounts of ionised bases (at least with phosphate buffers). Furthermore the retention of Li^+ (an indicator of silanol ionisation) does not increase with pH on some phases until at least pH 7 [5,6]. Thus, overloading of ionised silanols does not seem to be the cause of performance loss with increasing

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solute load (although this may not be true with older phases). It is possible that the repulsion of ionized solute molecules on the column surface contributes to the overloading process [3]. Amongst the evidence for this mechanism is that a similar overload also occurs for negatively charged species (ionised acids), and that this unusual overloading of charged species occurs also on polystyrene-based phases, which obviously have a completely different matrix to silica columns [7]. A different explanation proposed recently is that a small population (or populations) of (so-far unidentified) high energy yet unionised sites, which exist on the column surface along with a large population of weaker sites, can be rapidly overloaded by a wide range of (even neutral) solutes [8]. Even some combination of these two mechanisms could be envisaged.

Overloading seems to be reduced by mobile phase constituents that can form neutral ion pairs with the ionised solute, which reduces the number of charged species. Higher ionic strength mobile phases may also physically screen ions. The ionic strength of a 0.02 M solution of the weak acid formic acid ($pK_a = 2.75$) in water (pH ∼ 2.75) is only 1.9 mM—less than 10% of the total molar concentration of acid. Besides having low concentration, formate anions also have only weak ion pairing capability. In comparison, trifluoroacetic acid (TFA) is a much stronger acid ($pK_a = 0.43$) and its anion also has greater ion pair abilities; this acid reduces the effects of overloading [4]. Recently, significant ion pairing (which contributed to the reduction of overload) has been demonstrated for perchlorate and hexafluorophosphate, but even to a lesser extent for other inorganic anions such as chloride [9–11]. For the case of hydrophilic anions, the proportion of ion pairs may be increased substantially by their transfer from the mobile phase to the hydrophobic environment of the stationary phase. However, involatile anions are of little use in HPLC–MS. Even TFA is sometimes not favoured due to possible signal suppression effects in HPLC–MS; it can also prove undesirably persistent in the MS. Problems of overloading are greater in HPLC–MS, since low concentrations, even of "favourable" additives such as formic acid, are preferred due to reduced signal suppression effects [12,13].

In the present work, we have compared the peak shapes obtained for some basic drugs and peptides using mobile phases containing formic and acetic acid together with the same acid solutions adjusted with ammonia to a pH equivalent to their aqueous pK_a. Raising the pH in this way increases the concentration of anions to 50% of the molar concentration, increasing the opportunity for ion pairing to take place. The higher ionic strength may also have a favourable effect (see above). However, higher pH risks causing the ionisation of silanols, and another aim of the work was to investigate whether the onset of this process, giving kinetic tailing of peaks, might give an opposing detrimental effect on peak shape. We chose a hybrid silica/organic polymer (Xterra MS C18) for our investigation. This phase has no observable ion exchange capacity (using Li^+) below a pH of about 8 [5,6]. Although the behaviour of organic bases might not exactly replicate that of inorganic cations, the effect of silanol ionisation and detrimental ionic interactions on this particular phase should be much less likely to influence results at the pH values used in our study.

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 loop) 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 thermostat water bath. The column used was XTerra MS C18, 3.5 μm particle size, pore diameter 14 nm, surface area 176 m^2/g, 15 cm × 0.46 cm I.D. (Waters, Milford, USA); mobile phase flow was 1.0 cm^3/min. All peak shape measurements were made 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. The number of theoretical plates was calculated either using the half height method, or from the square of the first statistical moment divided by the second moment.

Column void volume was measured by injection of uracil. The Alberta peptide mix (RPS-10020) and individual peptide standards were obtained from the Alberta Peptide Institute (Edmonton, Canada); diphenhydramine and nortriptyline were obtained from Sigma–Aldrich (Poole, UK). Buffer additives were incorporated in both "A" and "B" solvents in gradient experiments to maintain a constant concentration throughout the analysis. Ionic strength calculations were performed using the PHoEBuS program (Analis, Orleans, France) using correction of activity coefficients according to the Debye/Hückel equation.

3. Results and discussion

Strongly basic drugs were chosen for our study; in common with the peptides (see below) they remain completely protonated in all the mobile phases used in our investigation. Initially, problems were experienced due to slow equilibration of Xterra MS with some of the mobile phases used, causing changes in retention with time for the injection of the same solution. However, equilibration in the mobile phase for 12 h overcame this difficulty, resulting in very reproducible retention for injection of solutions of the same concentration [14]. Figs. 1 and 2 show overlaid chromatograms of the strong bases diphenhydramine and nortriptyline (aqueous pK_a 9.3 and 10.3, respectively) for sample mass over the range 0.05–1.25 μg. The analysis was performed in 0.09% (w/v) formic acid (∼0.02 M, pH 2.75) and also in the same mobile phase adjusted to pH 3.75 with concentrated ammonia solution, bringing the solution pH up to the value of the pK_a for formic acid. This pH adjustment increases the ionic strength

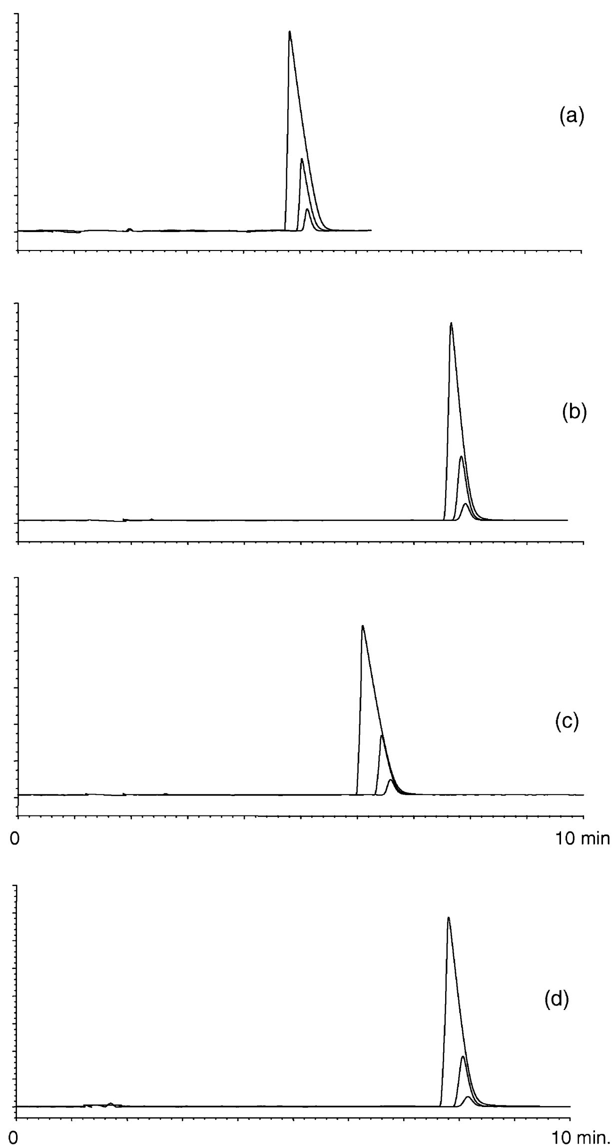


Fig. 1. Overlaid chromatograms for 1.25, 0.25 and 0.05 μg diphenhydramine on Xterra-MS. Mobile phase A–B (44:56, v/v): (a) A = 0.02 M formic acid in water pH 2.75, B = acetonitrile–0.04 M formic acid in water 50:50 (v/v); (b) A = 0.02 M formic acid in water pH 3.75, B = acetonitrile–0.04 M formic acid in water pH 3.75 with ammonia (v/v) 50:50; (c) A = 0.02 M acetic acid in water pH 3.75 with ammonia, B = acetonitrile–0.04 M acetic acid in water 50:50 (v/v); (d) A = 0.02 M acetic acid in water pH 4.75 with ammonia, B = acetonitrile–0.04 M acetic acid pH 4.75 in water 50:50 (v/v). Acetonitrile concentration 28% in each case. Detection UV at 215 nm. Temperature = 30 °C.

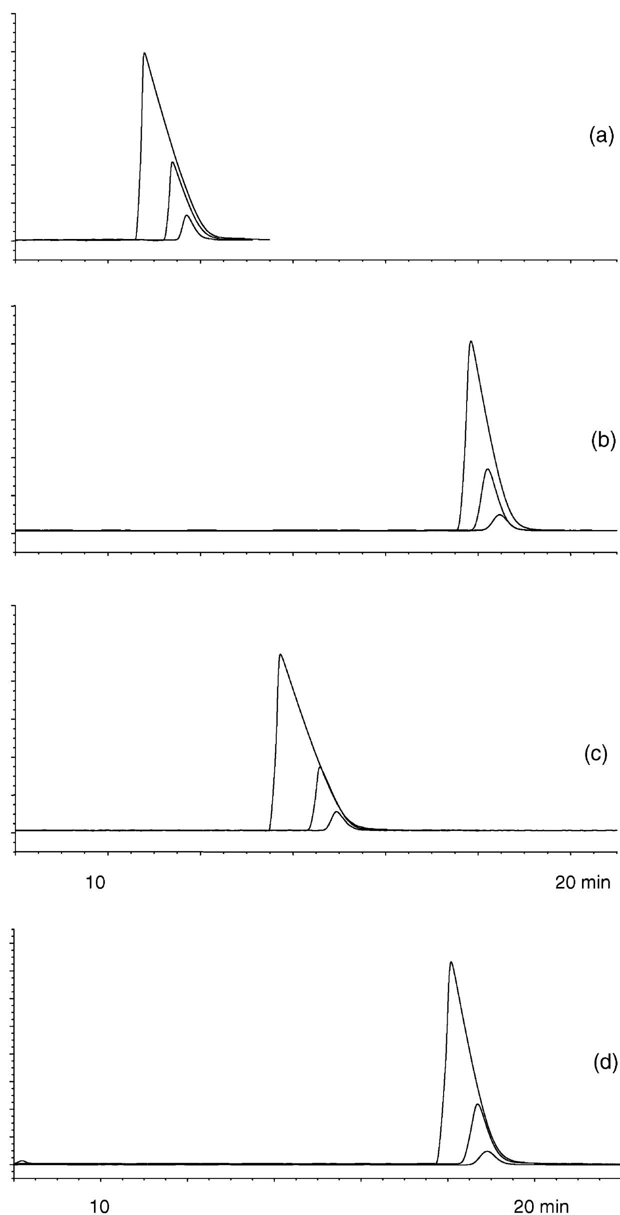


Fig. 2. Overlaid chromatograms for 1.25, 0.25 and 0.05 μg nortriptyline in mobile phases and using conditions as in Fig. 1.

of the solution from approximately 1.9–10 mM since ammonium formate is completely dissociated. However, the buffer capacity is not affected severely; formic acid itself is a reasonable "buffer" due to the large reservoir of hydroxonium ions which resists pH change. Thus any differences in behaviour are unlikely to be attributable to differences in the buffering capacity of the mobile phase. 0.02 M formic acid is very commonly used in HPLC–MS and indeed ready prepared solutions of this concentration are commercially available (Sigma–Aldrich). The chromatograms at pH 2.75 and 3.75 for both basic drugs with formic acid show evidence of overloading. As the sample mass is increased, retention is reduced, and peaks broaden, taking on the appearance of right-angled triangles, typical of overloading [15,16]. Nevertheless, with formic acid at pH 3.75, the peaks for highest sample mass of diphenhydramine and nortriptyline are noticeably less broad. Table 1 confirms these visual observations, indicating a considerable improvement in efficiency at pH 3.75, especially with larger sample loads. For example, at pH 2.75, efficiency is only 1460 plates for 1.25 μg of nortriptyline but 10,600 plates for 0.05 μg at the same pH. At pH 3.75, efficiency for 1.25 μg of nortriptyline is much higher at 6340 plates with 14,500 plates for 0.05 μg. Note that some improvement in

Table 1
Comparison of peak shapes and apparent saturation capacities using different buffers for basic drugs

	Mass (μg)	k	N	A_s	w_s (mg)	k	N	A_s	w_s (mg)
	Isocratic pH 2.75 formic					Isocratic pH 3.75 formic			
Nortriptyline	1.25	6.19	1460	7.9	0.5	10.9	6340	3.8	2.9
	0.25	6.60	4510	4.9		11.1	12300	1.9	
	0.05	6.80	10600	1.8		11.3	14500	1.3	
Diphenhydramine	1.25	2.22	1590	6.5	0.3	4.11	6190	3.5	2.2
	0.25	2.36	5320	3.4		4.23	12100	1.9	
	0.05	2.42	11500	1.8		4.27	14600	1.3	
	Isocratic pH 3.75 acetic					Isocratic pH 4.75 acetic			
Nortriptyline	1.25	8.16	1800	7.2	0.7	11.1	4900	3.8	2.3
	0.25	8.73	6520	3.5		11.5	9970	1.9	
	0.05	8.97	11600	1.9		11.6	11600	1.3	
Diphenhydramine	1.25	3.07	2100	5.3	0.6	4.21	4740	3.6	2.0
	0.25	3.29	6960	3.0		4.38	10500	1.8	
	0.05	3.39	12000	1.7		4.44	12300	1.4	

Mobile phases: see Fig. 1. Column efficiencies measured using the half-height procedure.

efficiency (from 10,600 to 14,600 plates) occurs even for the small 0.05 μg load when raising the pH from 2.75 to 3.75, with a reduction in asymmetry factor from 1.8 to 1.3. This result suggests that ionisation of silanols and resultant kinetic tailing has not occurred, in which case worse peak shape is expected for small sample mass as pH is raised. Furthermore, when ionised silanols are present, the initially low efficiency (at a given pH) may increase with increasing sample mass as silanols are "masked" by a portion of the sample itself—this effect is clearly absent here. Instead, it appears that some overloading still occurs even for 0.05 μg nortriptyline at pH 2.75 (see below for results with further reduced sample mass). Results for diphenhydramine are extremely similar to those for nortriptyline, with peaks for high load visibly less broad at pH 3.75 than at 2.75, confirmed by efficiency measurements in Table 1.

Overloading increases with increase in the retention factor k. This variable will lessen the apparent impact of improved loadability at pH 3.75, because k is increased at this higher pH and the effects are opposed. A possibility is to adjust the organic solvent composition to maintain k constant. However, we have preferred to compensate for variation in k by calculation of the apparent saturation capacity w_s, from

$$W_{\text{base}}^2 = \frac{16t_0^2(1+k_0)^2}{N_0} + \frac{6t_0^2k_0^2w_x}{w_s}$$

where W_{base} is the peak width at base, w_x is the sample mass of an overloaded peak, t_0 is the dead time, k_0 and N_0 are the retention factor and efficiency for a small sample mass (0.05 μg in the case of our experiments) [15–17]. w_s can then be calculated by additionally measuring the width of an overloaded peak (in our case for a 1.25 μg sample). w_s calculated in this way corresponds to an apparent saturation capacity, for example it may reflect the capacity of a subset of high energy sites rather than the column as a whole [8,15]. However, the direct probing of such sites alone is of interest, because being filled first, they dominate the performance of the column under the relatively low-load conditions of analytical separations. Nevertheless, this value is of less significance for preparative applications, where the determination of the total column saturation capacity by methods such as frontal analysis is indicated [8,15]. Table 1 shows these apparent saturation capacities of diphenhydramine and nortriptyline increase by a factor of about 6 (from 0.3 to 2.2 mg for diphenhydramine and from 0.5 to 2.9 mg for nortriptyline) when the pH of formic acid is increased from 2.75 to 3.75. Clearly, raising the pH gives a significant increase in performance of the Xterra phase, with increase loadability without silanol ionisation and resultant kinetic peak tailing.

We have shown recently that basic drugs and basic peptides overload in a rather similar fashion [4]. The Alberta mix is commonly used as a test of performance for peptide analysis; its detailed composition has been given previously [4]. It consists of four closely related synthetic peptides P1–P4 containing 1–4 basic lysine residues. In acidic mobile phases the peptides carry a charge of +1 to +4 due to protonation of the basic side chains; the C-terminals are amidated and the N terminals are acylated, so no charge arises from these groups. Peptides are almost inevitably separated using gradient elution due to the wide range of column sorption properties often encountered, and the large effect on retention of a small change in organic modifier content of the mobile phase [4]. Thus, we have used the peak capacity P as an estimate of performance instead of the efficiency given by the relationship

$$P = 1 + \frac{t_g}{1.699w_{0.5}}$$

where $w_{0.5}$ is the peak width at half height and t_g is the gradient time [18]. This equation strictly applies only to a Gaussian peak, and for tailing or overloaded peaks will give an optimistic value of the peak capacity, just as measurement of the peak width at half height exaggerates the plate count in isocratic separations. However, it is more difficult to measure peak widths reproducibly at base, as is

also the case in isocratic separations. The gradient program (1.25% acetonitrile min^{-1}) is typical of the rather slow gradients employed in peptide analysis due to the rapid change in their retention with organic modifier concentration. The Alberta mix was shown to give rather ideal behaviour, with each peptide giving virtually the same peak width (and thus peak capacity) for the same gradient slope under non-overloaded conditions when using phosphate buffers or TFA [4]. Fig. 3 shows overlaid chromatograms of the peptide mixture at normal strength (approx. 1.3–2.5 μg of each peptide injected) and diluted by 20 times (approx. 0.07–0.13 μg). As for the basic drugs, performance is visibly better at pH 3.75 using formic acid compared with pH 2.75, with narrower peaks

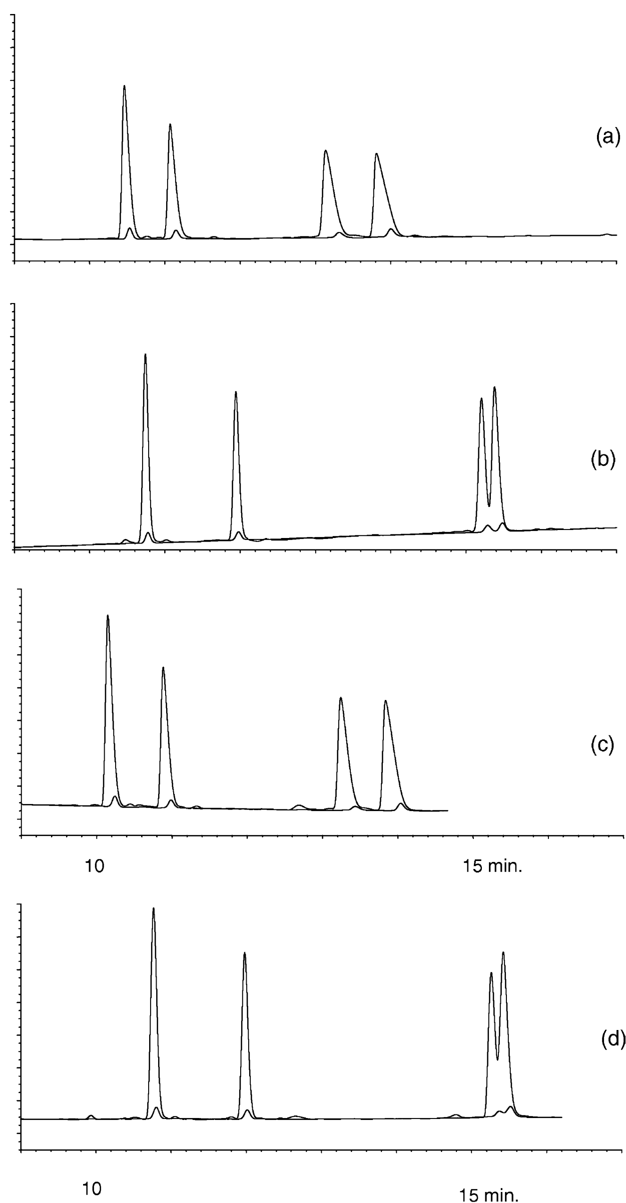


Fig. 3. Overlaid chromatograms for Alberta basic peptide mixture at normal strength and diluted 20×. Mobile phase gradients: 10% B to 60% B in 20 min (equivalent to 1.25% acetonitrile min^{-1}. Mobile phase solutions A and B as in Fig. 1.

produced for all peptides. The greatest differences are again shown for the more concentrated (normal strength) sample (see Table 2). For example, the peak capacity of P3 in the normal strength mix is only 100 at pH 2.75 but 178 at pH 3.75. Again, smaller but significant improvements in peak capacity are shown even for the diluted solutions of all of these peptides at the higher pH. For example, the peak capacity of dilute P4 was 168 at pH 2.75 but 194 at pH 3.75.

The improvement in performance for all basic solutes studied when raising the pH, even for very dilute solutions of the analyte is somewhat unusual, in that the increased ionisation of silanols and detrimental ionic interactions often cause significant decrease in efficiency at higher pH. However, the hybrid inorganic/organic polymer RP used in this investigation showed no evidence of these effects at pH 3.75, in confirmation of the results of other workers [5,6]. Our own studies [3], together with these reports, indicate that silanol ionisation may also not take place until quite high pH on *some* wholly silica based Type B phases. However, many Type B silica phases still show significant ionic interactions at low pH [19,20]. As noted above, raising the pH of formic acid from 2.75 to 3.75 caused increases in the retention of diphenhydramine, nortriptyline and the Alberta peptides, in addition to improving efficiency. It is possible in the absence of silanol ionisation (which could provide additional retention sites) that retention time increases are caused by the increased incidence of ion pairing, as the concentration of formate anions increases as the pH is raised. Increased ion pairing, whether it takes place in the mobile phase, followed by subsequent sorption of the (neutral) ion pair on to the surface of the phase, or in the stationary phase (initial sorption of the mobile phase anion, followed by interaction with the protonated solute or so-called “dynamic ion exchange”) also reduces the number of charged analyte species and the effects of overloading. Physical screening of ions by the higher ionic strength of formate at pH 3.75 could contribute to a reduction in overload. The retention of peptides is a weighted average of the retention of the paired and unpaired species. Fig. 3 shows that increases in retention in going from pH 2.75–3.75 formate are in the order P1 < P2 < P3 < P4 in line with their ability to form multiple ion pairs. Thus retention of P2 increases relative to P1 and retention of P4 increases relative to P3 at pH 3.75. Nevertheless, the degree of ion pairing with a hydrophilic anion such as formate is likely to be relatively small [10]. Thus, overloading still occurs, especially with peptides of higher charge. This result might be due to greater repulsion of highly charged unpaired ions. Alternatively, high-energy sites [8] within the column could be more easily overloaded by these multiply charged species.

If the above arguments are true, then 0.02 M acetic acid solution, adjusted to pH 3.75 with ammonia should give worse peak shapes than formate at exactly the same pH. Due to the higher pK_a of acetic acid (4.75), its ionic strength at pH 3.75 is only 1.8 mM/l (similar to formic acid at pH 2.75), and the number of anions available for ion pairing is low. Raising the pH of acetate solutions further to 4.75 should however,

Table 2
Comparison of peak shapes using different buffers for Alberta peptide mix

	Strength	t_R (min)	$w_{0.5}$ (cm)	Peak capacity	A_s	t_R (min)	$w_{0.5}$ (cm)	Peak capacity	A_s
	Formic gradient pH 2.7					Formic gradient pH 3.75			
P1	Normal	10.46	0.0995	178	2.2	10.74	0.0789	225	1.4
	20× diluted	10.53	0.0734	242	1.2	10.78	0.0734	242	1.1
P2	Normal	11.07	0.1080	164	2.5	11.95	0.0801	221	1.4
	20× diluted	11.15	0.0807	220	1.3	11.98	0.0770	230	1.3
P4	Normal	13.14	0.1541	116	3.0	15.26	0.0995	178	1.6
	20× diluted	13.32	0.1056	168	1.4	15.29	0.0916	194	1.5
P3	Normal	13.81	0.1784	100	3.9	15.44	0.0995	178	1.8
	20× diluted	14.00	0.0934	190	1.6	15.5	0.0886	200	1.5
	Acetic gradient pH 3.75					Acetic gradient pH 4.75			
P1	Normal	10.16	0.1007	176	1.9	10.77	0.0886	200	1.3
	20× diluted	10.24	0.0764	232	1.0	10.80	0.0837	212	1.1
P2	Normal	10.89	0.1074	165	2.1	11.98	0.0910	195	1.3
	20× diluted	10.99	0.0801	221	1.1	12.02	0.0843	210	1.1
P4	Normal	13.25	0.1383	129	2.5	15.24	0.1031	172	1.6
	20× diluted	13.44	0.1092	163	1.4	15.32	0.1152	154	2.7
P3	Normal	13.85	0.1638	109	3.0	15.42	0.1104	161	1.5
	20× diluted	14.04	0.0934	190	1.3	15.52	0.0971	183	1.4

Mobile phases: see Figs. 1 and 3. Peak capacities calculated for a gradient time of 30 min.

give rise to improvements in performance, as long as silanol ionisation and kinetic tailing does not occur. 0.02 M acetic acid adjusted to pH 4.75 with ammonia has ionic strength 10 mM/l (the same as 0.02 M formic acid adjusted to pH 3.75) and again by the same argument should produce similar results to formate at pH 3.75. Figs. 1–3, show that these predictions are correct. Serious overloading effects are shown for nortriptyline with acetate at pH 3.75, with only 1800 plates for a 1.25 μg sample compared with 11,600 plates for 0.05 μg. Similarly 1.25 μg diphenhydramine gave 2100 plates but 12,000 plates for a 0.05 μg sample. Apparent saturation capacities for diphenhydramine and nortriptyline at pH 3.75 with acetate were 0.6 and 0.7 mg, respectively, considerably lower than for formate at pH 3.75 but similar to results for formic acid at pH 2.75. Comparable results were also obtained for the peptide mixture (Fig. 3, Table 2). For example, the peak capacity of high and low loads of P3 using pH 3.75 acetate were 109 and 190, respectively, which are very similar to those obtained with pH 2.75 formic acid (100 and 190, respectively) but considerably worse for high sample load than with pH 3.75 formic acid (178 and 200, respectively).

The retention of both the drugs and peptides with acetate at pH 4.75 was similar to formate at pH 3.75, but increased compared with acetate at pH 3.75, which can be attributed to the increased ion pairing taking place as the concentration of acetate ions increases with pH. However, Tables 1 and 2 indicate slightly reduced *absolute* values of efficiency with pH 4.75 acetate compared with pH 3.75 formate. Although the *relative* decrease in performance with load is similar (note *k* values are similar). This difference can be attributed to some decline in performance which occurred during a period of column storage (3 months) in acetonitrile/water before this last set of results was obtained. Re-evaluating in 0.02 M formic acid pH 2.75 indeed showed a drop in absolute efficiency to about 85% of its original value over this period. Apparent saturation capacities for diphenhydramine and nortriptyline were 2.0 and 2.3 mg, respectively which indicates improvement over performance with acetate at pH 3.75. The behaviour of peptides was also similar with acetate at pH 4.75 to formate at pH 3.75. For example, peak capacity for high load of P3 with acetate at pH 4.75 (154) is 90% of that for low load (172). For pH 3.75 formate, peak capacity for high load of P3 (178) is 89% of its value at low load (200) indicating very similar behaviour. However, peak capacity for the dilute solution of the most highly charged peptide P4 (154) was *lower* than that for normal working strength (172) when using pH 4.75 acetate buffer. This apparently anomalous result was confirmed using a brand new column of exactly the same type (results not shown) and thus cannot be attributed to degradation in the storage period. This highly charged peptide may indeed be an exceptionally sensitive indicator of silanol effects [21] which have begun to appear at pH 4.75. Note that the pH of the solution is raised by the presence of the organic solvent, and the ${}_{w}^{s}$pH at this point in the gradient is around 5.3. However, the pK_a of the silanols (which are weak acids) is likely to be raised too, so the dissociation of the silanols is probably not affected much by the presence of the organic solvent. Silanol effects were observed for P4 on a conventional Type B RP silica at somewhat lower pH [4], so this result still indicates a high degree of inertness of the Xterra phase.

Figs. 4 and 5 show the loss in retention time and efficiency with increasing sample mass of diphenhydramine for formic acid at pH 2.75 and acetate at pH 4.75 using the replacement column and a wider range of sample mass. These data

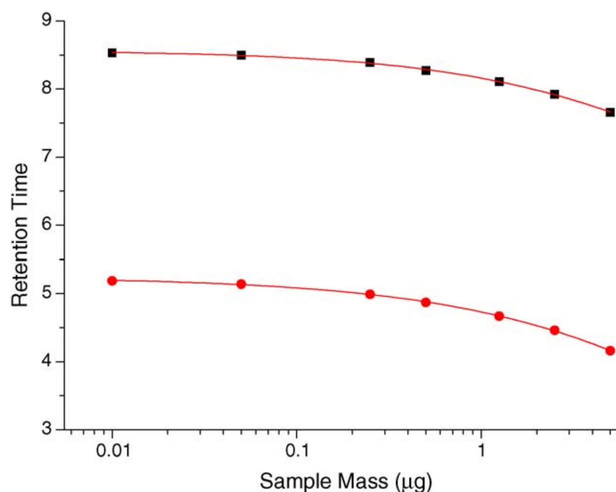
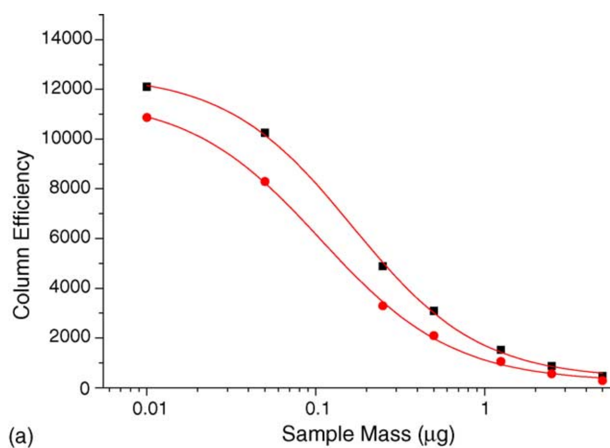


Fig. 4. Plot of retention time vs. sample mass for diphenhydramine on (replacement) Xterra MS using (lower plot) formic acid pH 2.75 as in Fig. 1(a) and (upper plot) acetic acid pH 4.75 as in Fig. 1(d).



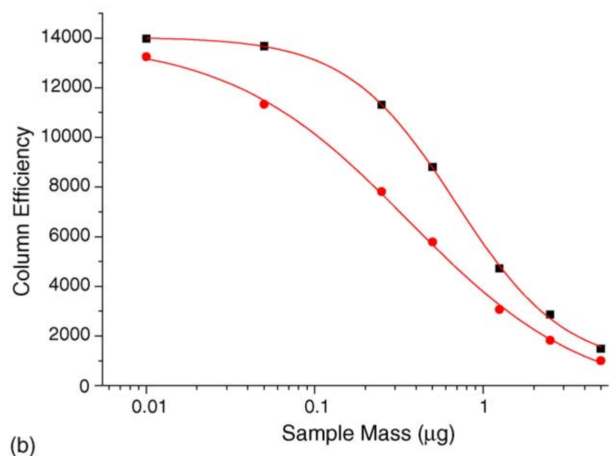


Fig. 5. Plots of efficiency (N) vs. sample mass for diphenhydramine on (replacement) Xterra MS using (a) mobile phase formic acid pH 2.75 as in Fig. 1(a) and (b) mobile phase acetic acid pH 4.75 as in Fig. 1(d). In both Fig. 5(a) and (b) upper plot N measured using half height method, lower plot N using statistical moments method.

yielded similar apparent saturation capacities to the original column indicating good column to column reproducibility of the overloading effect. At pH 2.75 with formic acid, measurable differences in retention time can be seen even between 0.01 and 0.05 μg samples. Thus, it would have been preferable to use even lower sample masses that 0.05 μg to calculate N_0 for the case of pH unadjusted formic and acetic acid mobile phases. For our data however, the difference in calculated values of apparent saturation capacity produced was not large. In agreement with previous results [3], about one third of the initial efficiency was lost for a sample mass of 0.1 μg (Fig. 5a), whether the efficiency is measured by the half-height or the statistical moments procedure. The latter method gives a more realistic view of efficiency for non-Gaussian peaks, although is less reproducible at low signal to noise ratio. Nevertheless, the plots show exactly the same trends. Changes in both retention and efficiency are visibly less when using acetate at pH 4.75 compared with formic acid at pH 2.7 using either method of calculating efficiency. The continuous decrease in retention is predicted by theory because the sorption of material must change the accessible volume of the stationary phase and hence the phase ratio and retention properties. However, there is a popular conception that overload only occurs once some threshold value of sample mass is exceeded. In the present work with weak acid mobile phases, measurable changes in retention and efficiency have been shown with sample mass even in the region near the detection limit of the solutes. Nevertheless, these effects may still be observed using phosphate buffers, albeit to a lesser extent [3].

Finally, Fig. 6 plots the predicted efficiency (calculated from the apparent saturation capacity determined from a single overloaded injection (see above [22]) together with experimentally measured efficiency (using the half height method). Rough agreement between calculated and measured results is

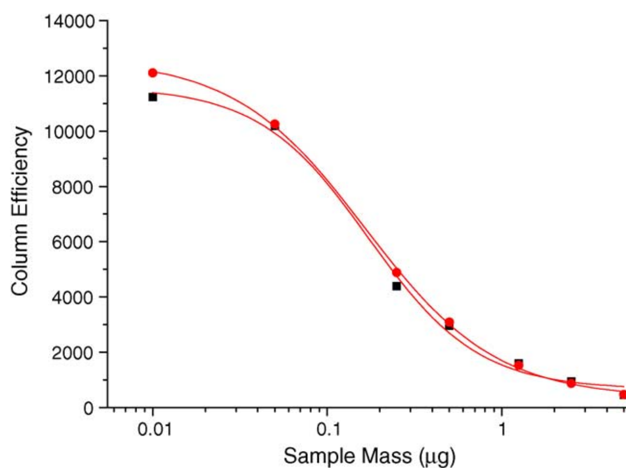


Fig. 6. Plots of experimentally measured efficiency (upper plot) and efficiency predicted by calculation of apparent saturation capacity (lower plot) against sample mass of diphenhydramine using formic acid pH 2.75 mobile phase as Fig. 1(a).

shown. This figure indicates that the equation could be used to predict approximately column efficiencies at different loads. However, we continue to seek alternative simple measures of overload in analytical chromatography.

4. Conclusions

The effects of overload are particularly serious for basic drugs and peptides in low ionic strength weak acid buffers often used in HPLC–MS. For example, efficiency decreases by almost a third as sample load is increased from 0.01 to 0.1 μg of diphenhydramine when using 0.02 M formic acid pH 2.75 on a 0.46 cm I.D. column. Overloading is a continuous process with no "threshold" value of sample mass above which the phenomenon occurs, as is commonly believed. However, a practical recommendation for HPLC–MS is that overload can be reduced by adjustment of weak acids such as formic acid to higher pH, providing column silanol ionisation does not occur. pH adjustment, e.g. to the pK_a of the acid additive increases the ionic strength, giving increased possibility for ion pairing with mobile phase anions and possibly increased physical screening of solute ions. A greater proportion of neutral ion pairs could either reduce mutual ionic repulsion [3] or limit the interaction of ionic solutes with high energy sites [8]. Use of a pH close to the pK_a of the acid also gives optimum buffer capacity.

No detrimental ionic interactions with dissociated silanols were noted using the Xterra phase at pH 3.75 with formic acid. For acetic acid, improvements in loadability were noted at higher pH (4.75) due to the higher pK_a of acetic acid. Caution is necessary even with the Xterra phase at this higher pH, since it is possible that some silanol ionisation takes place which could give detrimental interactions for highly charged species.

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