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Comparison of peak shapes obtained with volatile (mass spectrometry-compatible) buffers and conventional buffers in reversed-phase high-performance liquid chromatography of bases on particulate and monolithic columns

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Abstract

Retention factor, column efficiency and asymmetry factor were recorded for nine basic compounds on a number of RP-HPLC columns using phosphate and a variety of (MS-compatible) volatile mobile phase buffers of acid and neutral pH, in order to assess any effects of the buffer on performance. With formic or acetic acid, some phases gave partial or complete solute exclusion effects (reduced or negative k) compared with results using phosphate buffers at low pH. Despite its possible suppression of mass spectrometer sensitivity, trifluoroacetic acid was useful in enhancing retention times of relatively hydrophilic protonated bases, due to ion-pair effects. Peak shape was relatively poor on some pure silica-based ODS phases at pH 7 compared with results at acid pH. At low pH and at pH 7, ammonium and potassium phosphate gave very similar k, but the former may be preferable due to its volatile cation. Improved peak shapes, attributed to superior silanol masking effects, were obtained with ammonium phosphate at pH 7, but not at acid pH. Ammonium acetate gave acceptable peak shape at pH 7, but due to very limited buffer capacity, poor results were obtained for solutes having a pK_a close to the mobile phase pH. Due to its instability, ammonium hydrogen carbonate is not a viable alternative buffer at pH 7.

Keywords: Peak shape; Buffer composition; Stationary phases, LC

1. Introduction

The poor peak shapes obtained from many pharmaceuticals and other biomedically important compounds which have basic properties continues to be a problem in their analysis by HPLC in the RP mode [1]. Underivatised silanol groups existing on the surface of RP columns are considered to be the cause of the broad and tailing peaks which often result.

Due to recent advances in instrumentation together with significant reductions in the cost, LC–MS techniques have become increasingly popular. In a carefully controlled study [2], it was shown that short-term repeatability of retention times measured by HPLC–UV or HPLC–MS (using atmospheric pressure chemical ionisation, APCI) gave equally good precision. The excellent selectivity of the MS technique, and in some cases superior limits of detection, have contributed to it replacing existing UV detection methods for some applications, especially in the pharmaceutical industry. For example,

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extensive sample clean-up may not be required if the detection system can be operated so as not to sense interfering matrix compounds. Similarly, much faster analysis may be achieved if overlapping peaks can be separately visualised by the MS detection system [3]. Nevertheless, precision of peak area and column efficiency measurements reported in one study were almost three times worse by HPLC–MS than HPLC–UV [2].

In many cases, direct transfer of mobile phases developed for HPLC–UV is not possible, for example due to loss of sensitivity, or other interferences experienced due to mobile phase components when using MS [4,5]. In particular, the use of involatile buffer components such as phosphate, which is popular in HPLC–UV, may cause practical difficulties due to the build up of residues in the source. Frequent cleaning may be necessary, or other more serious problems can result. However, some types of interface such as orthogonal electrospray are somewhat more tolerant of involatile buffers [6]. Some organic buffers or additives such as trifluoroacetic acid can result in MS signal suppression in the positive ion mode and total absence of signal in the negative electrospray ionization (ESI) mode [4,7]. Furthermore, differences in peak shape and retention of basic compounds have been encountered when using different buffer compounds. It has been shown in general terms that results using different buffers may be poorly correlated in terms of tailing factors produced for test compounds [8]. However, no detailed studies exist which evaluate any detrimental change in chromatographic performance when substituting volatile buffers for phosphate, or give recommendations or cautions on the use of particular buffers.

In the present study, we have compared *k*, column efficiency and asymmetry factor of several ''new generation'' RP columns using different volatile and involatile buffers at the same pH. We studied several conventional microparticulate columns and a monolithic phase. Our studies have been confined to the evaluation of chromatographic performance using UV detection, rather than a combined study including MS effects such as signal suppression. Since poorer reproducibility of chromatographic performance measurements may be obtained in HPLC–MS compared with HPLC–UV [2], separate evaluation of column and detector factors may actually be beneficial.

2. Experimental

The HPLC system used consisted of P200 pump, UV 100 detector (1 μl cell) operated at 254 nm or 215 nm (Thermo Separation Products, San Jose, USA) and 7725 valve injector with 2 μl loop (Rheodyne, Cotati, USA) together with a model 2000 data station (Trivector, Bedford, UK). Results for one set of measurements only (Eclipse column) were obtained on an Agilent 1100 system and Chemstation using the same injector and a similar 1 μl detector cell. Nevertheless, the same instrument was always used to evaluate a particular phase; no direct comparisons in the present study have been drawn between the results for the Eclipse column and those for the other phases. Connections were made with minimum lengths of 0.01 cm I.D. tubing, in order to further minimise extra-column effects. Temperature control in both systems was achieved by immersing the column and injector in a thermostatted water bath. A 3 m×0.05 cm 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 before delivery to the injector and column. Column efficiency was determined using the half height (N) and the Dorsey–Foley procedure [9], $N_{df} = 41.7[t_r/w_{0.1}]^2/[A_s + 1.25]$ which has been shown, also by others, to give a reasonable estimate of true efficiency for asymmetric peaks [10]. 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 columns used are detailed in Table 1. Preparation of buffers was as described previously with pH measured before addition of organic solvent [11]. All results were the mean of at least duplicate injections of single compounds, to prevent interference effects which can occur using peak shape measurement with mixtures [12,13]. Sample sizes of 200 ng were used in an effort to minimise overloading effects [14]. Column void volume was measured by injection of uracil.

Table 1
Columns used in this study; data supplied by manufactures

Column	Manufacturer	Dimensions (cm) (length×I.D.)	Surface area (m^2 g^{-1})	% Carbon	Pore diameter (nm)
Symmetry 100 (C_{18})	Waters	25×0.46	341	19.9	9
Symmetry 300 (C_{18})	Waters	25×0.46	112	8.5	24
Chromolith (C_{18})	Merck	10×0.46	300	18	13 (mesopore)
Discovery (C_{18})	Supelco	25×0.46	194	12.6	19
Eclipse XDB (C_8)	Agilent	25×0.46	180	7.6	8

3. Results and discussion

3.1. Comparison of buffers at low pH

Table 2 shows a comparison of retention factor (k), plates per metre measured based on N and N_{df}, and A_s for a variety of C_{18} RP columns tested with different buffers (0.02 M) of the same pH (all pH 2.7). However, trifluoroacetic acid (TFA) was used, within the concentration range and pH usually employed (0.9 g l^{-1}, pH 2.3) without further pH adjustment to avoid introduction of extraneous components. Both potassium phosphate and ammonium phosphate were studied, the latter having a volatile cation which renders it more suitable for use with some HPLC–MS interfaces. Each column was evaluated with the same nine basic test compounds which we have used previously [12,13]. These cover a range of pK_a values and have different stereochemistries. A single basic probe is unsatisfactory, since a given phase may give relatively good performance for one compound, but poor performance for another. Principal components analysis is a useful aid in the selection of suitable compounds [15]. In the present study, we used a relatively low concentration of buffer (usually 0.018 M overall) to simulate conditions generally employed in HPLC–MS. The concentration of buffers used in practice may be even lower than this value. For example, loss of sensitivity was found to occur in an electrospray system as the concentration of formic or acetic acid was increased over the range 0–100 mM, with the effect being more serious for the more commonly used formic acid [4,5]. However, we used this somewhat higher concentration to avoid the possibility of poorer reproducibility in chromatographic performance, which is important in the present study.

Individual and mean retention factors were very similar using potassium phosphate and ammonium phosphate buffers at pH 2.7 on a given phase for Symmetry 100, Discovery and Chromolith (the monolithic phase), as shown in Table 2. In each case, N, N_{df} and A_s on a given phase with either buffer were also almost identical. The performance of Discovery and Symmetry 100 with most compounds was satisfactory, with $A_s = 1.5$ or less for most solutes; however, somewhat greater tailing was found for nortriptyline. In comparison, Chromolith gave more asymmetric peaks than either of the conventional particulate phases using the same phosphate buffers, with mean A_s around 2.0. This higher value can be attributed partially to increased tailing found even for neutral compounds such as benzene [which gave $A_s = 1.8$ with acetonitrile–water (40:60, v/v) at the same flow-rate], compared with the particulate phases which all gave A_s close to unity. The mean column efficiency of the monolith calculated using N was greater than for the conventional phases (approx. 115 000 plates m^{-1} compared with about 90 000 plates m^{-1}). However, use of N_{df}, which takes into account peak tailing, yielded a lower efficiency for the monolith (about 50 000 plates m^{-1}) compared with the conventional phases (65 000 to 70 000 plates m^{-1}). Despite this increased tailing, the monolith has significant advantages when operated at high flow velocity, as shown previously, due to the flatness of the Van Deemter plots obtained [16]. Chromolith also showed lower mean k in all buffers compared with the particulate columns, which can be attributed to the sparser occupation of the column by the stationary phase.

Table 2
Evaluation of columns at low pH. Mobile phases: a = acetonitrile–0.020 *M* KH_2PO_4 pH 2.7, b = acetonitrile–0.020 *M* NH_4HPO_4 pH 2.7, c = acetonitrile–0.020 *M* formic acid pH 2.7, d = acetonitrile–0.9 g l^{-1} TFA pH 2.30; all acetonitrile–buffer (10:90, v/v) except for diphenhydramine and nortriptyline (28:72, v/v); flow-rate 1 ml min^{-1}; column temperature: 30 °C. All *N* values in plates/m

	Pyridine				Procainamide				Nicotine				Amphetamine				Codeine			
	k	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s
Symmetry C_{18}																				
a	−0.11	112 100	84 000	1.24	0.35	87 900	80 100	1.11	−0.10	99 500	83 100	1.20	2.62	91 300	71 700	1.51	1.60	76 300	68 000	1.20
b	−0.11	110 700	78 400	1.34	0.28	85 100	77 200	1.12	−0.07	86 900	71 400	1.13	2.43	91 500	71 400	1.50	1.55	78 600	70 900	1.18
c	−0.28	117 600	74 100	1.41	−0.21	103 000	87 000	1.15	−0.31	127 800	102 700	1.17	0.39	105 400	93 900	1.12	0.14	78 400	70 400	1.15
d	−0.08	101 300	76 300	1.26	0.34	75 700	73 000	1.02	−0.07	86 800	80 900	1.02	4.88	86 000	70 700	1.35	3.08	81 800	76 100	1.15
Discovery C_{18}																				
a	−0.06	111 700	75 800	1.23	0.25	89 200	62 800	1.26	−0.03	97 300	66 700	1.29	2.01	88 500	67 700	1.43	1.25	79 600	64 100	1.18
b	−0.04	112 400	77 600	1.20	0.32	85 500	60 900	1.26	−0.01	97 800	69 500	1.25	2.16	88 400	63 600	1.43	1.42	81 900	64 700	1.21
c	−0.16	110 200	80 600	1.10	0.07	72 000	55 900	1.29	−0.15	104 800	76 400	1.08	1.32	49 100	21 300	2.80	0.88	69 900	41 800	1.87
Monolith																				
a	−0.03	148 300	57 900	2.00	0.18	143 000	70 800	1.80	−0.01	153 800	67 100	1.86	1.45	97 500	36 700	2.42	0.91	113 300	57 400	1.80
b	−0.04	148 000	58 100	1.99	0.21	142 500	69 900	1.77	−0.02	154 500	67 500	1.84	1.46	94 000	32 800	2.56	0.98	112 700	57 900	1.81
c	−0.05	151 000	76 000	1.63	0.14	125 900	80 900	1.29	−0.04	144 000	82 700	1.40	1.07	35 400	13 800	2.29	0.58	74 000	34 200	1.49
d	0.02	145 000	46 400	2.22	0.29	137 100	75 000	1.64	0.06	92 400	51 300	1.66	2.50	81 800	29 600	2.57	1.74	101 800	56 000	1.69
Symmetry 300																				
a	−0.06	76 900	56 900	1.40	0.22	63 200	51 300	1.30	−0.03	69 400	55 500	1.31	1.73	69 800	55 500	1.50	1.08	63 800	52 500	1.33
c	−0.08	81 100	58 600	1.47	0.19	61 900	45 100	1.56	−0.05	73 100	58 100	1.34	1.61	33 800	15 000	3.37	1.01	48 000	30 900	2.07

	Quinine				Benzylamine				Diphenhydramine				Nortriptyline				Mean column			
	k	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s
Symmetry C_{18}																				
a	4.82	77 200	66 200	1.27	0.42	102 700	88 100	1.24	2.33	78 700	55 800	1.59	5.89	80 200	56 000	1.70	1.98	89 600	72 600	1.34
b	5.13	77 000	66 700	1.25	0.42	98 200	86 000	1.20	2.33	80 200	58 300	1.56	5.94	80 900	56 500	1.69	1.99	87 700	70 800	1.33
c	0.96	60 600	51 700	1.14	−0.16	120 500	106 600	1,08	0.40	76 700	61 700	1.25	1.40	70 100	42 300	1.70	0.26	95 600	76 700	1.24
d	7.88	76 900	69 100	1.23	0.90	91 900	82 300	1.17	3.28	75 700	64 800	1.30	8.88	76 000	65 500	1.32	3.23	83 600	73 200	1.20
Discovery C_{18}																				
a	3.60	82 700	61 500	1.29	0.37	104 600	74 200	1.33	2.79	90 600	63 200	1.43	6.87	74 300	51 500	1.55	1.89	90 900	65 300	1.33
b	5.10	82 600	62 300	1.29	0.43	96 700	69 000	1.32	2.37	85 500	59 700	1.48	6.09	80 100	55 700	1.51	1.98	90 100	64 800	1.33
c	2.90	47 900	24 400	2.30	0.15	79 400	45 700	2.05	1.55	39 000	18 300	2.91	4.62	33 400	14 100	3.12	1.24	67 300	42 100	2.06
Monolith																				
a	3.69	88 700	43 700	1.97	0.26	131 200	54 800	2.07	1.51	101 800	51 900	2.02	3.74	80 600	41 700	2.13	1.30	117 600	53 600	2.01
b	3.83	83 700	38 200	2.04	0.25	132 400	55 000	2.12	1.56	94 600	45 400	1.96	3.78	78 100	40 600	1.96	1.33	115 600	51 800	2.01
c	2.50	57 100	31 000	1.10	0.16	91 100	39 500	2.01	1.04	28 900	13 400	1.48	2.58	19 900	9280	1.51	0.89	80 800	42 300	1.58
d	4.81	77 900	35 000	1.91	0.56	118 100	48 800	2.13	2.13	88 200	48 000	1.59	5.16	67 800	36 300	1.68	1.92	101 100	47 400	1.90
Symmetry 300																				
a	3.70	61 000	11 700	1.50	0.31	74 500	59 600	1.40	2.51	60 600	43 700	1.67	6.57	60 700	42 100	1.79	1.78	66 700	47 600	1.47
c	3.57	45 480	23 300	2.50	0.25	54 300	33 700	2.14	2.73	24 700	7960	4.39	6.99	21 800	5890	4.95	1.80	49 400	31 000	2.64

Generally, it can be concluded that ''partially volatile'' ammonium phosphate buffers can be substituted for potassium phosphate in HPLC–MS at low pH with very little change in peak shape or retention.

Some apparent exclusion of pyridine and nicotine, which elute before uracil giving negative *k* values, was found for all phases using phosphate buffers. Furthermore, on Discovery and Chromolith, use of formic acid instead of phosphate caused increase in the exclusion of these compounds (higher negative *k*). Accompanying this result was a drop in retention for non-excluded compounds, resulting in reduction of mean *k* from about 1.9 to about 1.2 on Discovery and from 1.3 to 0.9 on Chromolith. Using Symmetry 100, these exclusion effects were much more serious. In addition to much greater exclusion of pyridine and nicotine ($k=-0.28$ and -0.31, respectively), procainamide and benzylamine were also excluded when using formic acid. Retention factors for all compounds dropped considerably, e.g. nortriptyline from $k=5.9$ in phosphate buffers to 1.4 in formic acid at the same pH. Mean *k* for all compounds using formic acid on Symmetry 100 was only 0.26 compared with 2.0 using phosphate buffers. Clearly, substitution of phosphate by formic acid caused major changes, and few of the test bases could be separated from one another in the latter mobile phase due to low retention. Reducing the acetonitrile content to increase *k* might risk alkyl-ligand collapse and further loss of retention for those solutes analysed in 10% acetonitrile which is already a low concentration. Whereas Discovery and Chromolith showed considerable decreases in mean efficiency using formic acid compared with phosphate buffers, Symmetry 100 showed a small efficiency increase. However, inspection of the results reveals this increase to be associated with those solutes excluded or further excluded from the stationary phase in formic acid. For example, benzylamine gives about 100 000 plates m^{-1} in phosphate buffers (when not excluded) but about 120 000 plates m^{-1} in formic acid, in which it yields a negative *k*. It is not unreasonable to assume that band broadening processes should be reduced for those solutes not able to enter the stationary phase structure. These differences might not be revealed using instruments with larger extra column volume than that in our optimised systems. However, these efficiency increases for excluded solutes are hardly of practical importance. Note also that as shown previously, there is not a simple negative correlation of asymmetry values and column efficiency (i.e. column efficiency does not drop exactly in line with increase in asymmetry factor [15]. For example, mean efficiency decreases for Chromolith in formic acid compared with phosphate buffers, but the mean asymmetry factor also decreases. We believe there is a danger in the use of asymmetry parameters alone, since they do not record peak broadening factors, or complex peak shapes in which, for example, simultaneous fronting and tailing occurs. In addition, unlike column efficiency measurements, they have no fundamental significance in chromatographic theory.

Trifluoroacetic acid caused increase in mean *k* for the test solutes on Symmetry 100 and Chromolith compared with phosphate buffer. Note that a lower pH (2.3) compared with phosphate buffers (2.7) was used, and this might be expected to produce a *decrease* in retention for those solutes containing weakly basic groups. The increase in *k* can be attributed to the ion-pair effect of trifluoroacetate [7]. Retention increase is beneficial for those protonated bases having low retention at acid pH. Column efficiencies for Symmetry 100 and Chromolith with TFA were similar to those using phosphate buffers. About half of the small drop in the mean efficiency of Chromolith from approximately 115 000 plates m^{-1} using phosphate buffers to 100 000 plates m^{-1} using TFA can be attributed to the drop in plate count of nicotine. Nicotine is excluded from the monolith in phosphate buffers, but not in TFA (see above). Thus, from a consideration of chromatographic performance alone, TFA is a useful substitute for phosphate buffers. However, its MS signal suppression and possible contamination of MS instruments due to its long persistence, may preclude its use on other considerations [4,5], unless it can be removed from the eluent, or its effects reduced, prior to the detection stage [17].

3.2. Investigation of exclusion effects on Symmetry 100

The difference in the degree of exclusion of the test solutes using acidic mobile phases appears to be related to the differences in pore size of the various

materials (Symmetry 9 nm, Chromolith 13 nm, Discovery 19 nm) with the smallest pore phase giving the most significant effect. To check this hypothesis, we repeated the column tests using a large pore Symmetry 300 C_{18} phase. Fig. 1 shows graphically the large reduction in *k* for individual test compounds using formic acid compared with potassium phosphate buffers at pH 2.7 on Symmetry 100. In contrast, Symmetry 300 showed only very small differences in *k* using these two buffers. Only pyridine and nicotine continued to show very small negative *k* on Symmetry 300 in either phosphate or formate buffers. For Symmetry 300, efficiencies using formic acid were lower than with phosphate buffers, and asymmetry was increased, a result similar to that obtained with Discovery and Chromolith, now exclusion effects had been largely removed. It is possible that improved peak shapes with phosphate buffers compared with formic acid (in the absence of exclusion) are due to the competitive ion interaction effects of buffer K^+ or NH_4^+ with a small number of highly acidic silanols, which remain even on these high purity phases at low pH. The small number of residual silanols, however, may not contribute greatly to retention on these high purity phases, as shown by similar *k* for solutes in both mobile phases for Symmetry 300. Note that the relatively small concentration of organic solvent used in the mobile phase is not likely to give rise to large pH differences after combination of different aqueous buffers with organic solvent [18]. However, pH differences after mixing may need to be considered in mobile phases of increased organic content.

Some workers [19,20] have observed an unexpected increase in retention of some aniline and pyridine derivatives as mobile phase pH was lowered below 3. They attributed this effect to disruption of the sheath of water molecules surrounding the basic analyte (Hofmeister effect) by the counter ions of the acidic titrant used to adjust pH. As the concentration of counter anion was increased, it was proposed that decrease in analyte solvation increased hydropho-

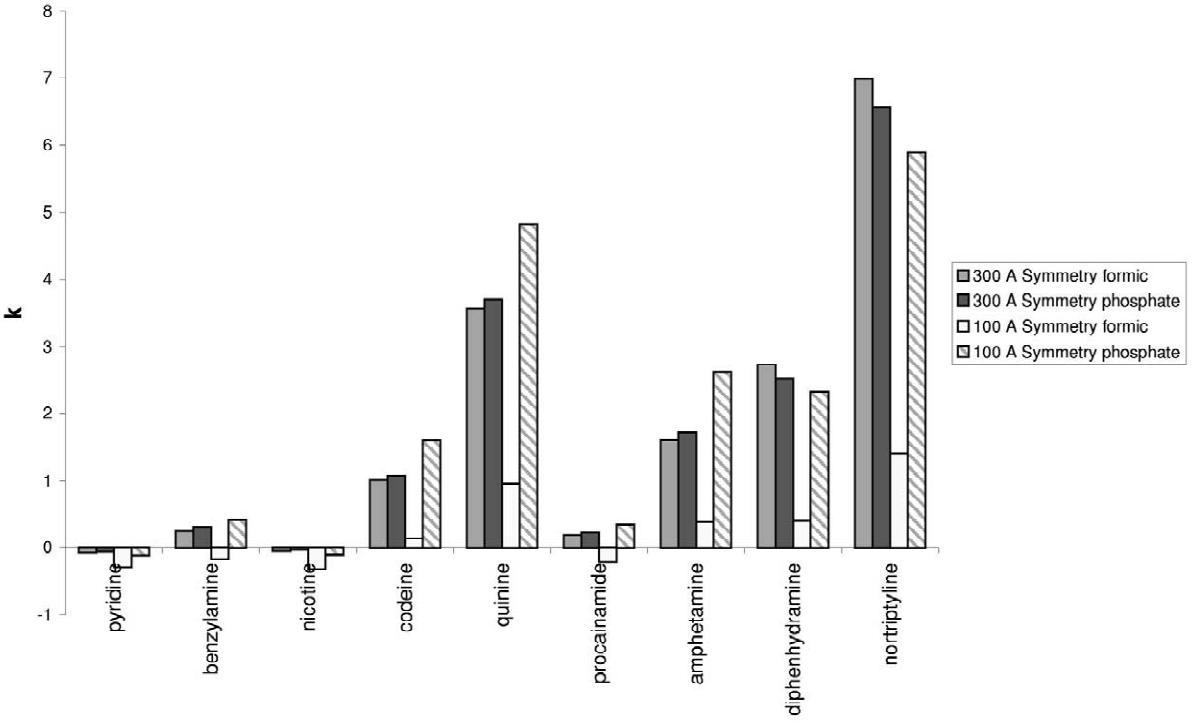

Fig. 1. Comparison of *k* using 300 and 100 Symmetry C_{18} columns. Formic acid mobile phase acetonitrile–0.02 *M* formic acid pH 2.7 (10:90 v/v, except diphenhydramine and nortriptyline 28:72, v/v), phosphate mobile phase acetonitrile–0.02 *M* phosphate buffer pH 2.7 (10:90, v/v except diphenhydramine and nortriptyline, 28:72, v/v).

bicity, leading to increased interaction with the hydrophobic stationary phase and thus increased retention. The largest retention changes were reported over the range 0–0.02 *M* counteranion concentration, after which the effect apparently levelled off, with little change in retention caused by increases above 0.03 *M*. It was proposed that the Hofmeister effect increased in the order $(H_2PO_4)^- < (CF_3COO)^- < (ClO_4)^-$, and that the same degree of desolvation was achieved at much lower concentrations of the anion at higher organic solvent concentrations. This factor was attributed to the contributory effect of solvents like acetonitrile to analyte desolvation. ''Salting out'' is a technique which has been used in the purification of proteins for many years [21]. Buffer anions, such as phosphate or sulfate, and cations such as ammonium or potassium, appear to desolvate proteins, causing them to precipitate, if the concentration of salt is sufficiently high. Such interactions are important in hydrophobic interaction chromatography, in which proteins can be eluted from a RP using a negative salt gradient.

In order to investigate exclusion effects further, we compared the retention of the test solutes using ten different acetonitrile–buffer (10:90, v/v) combinations, of various composition and concentration. The results, including those for 0.02 *M* potassium phosphate (buffer 1), formic acid pH 2.7 (buffer 3) and TFA (buffer 10), obtained previously, are shown in Fig. 2. Increasing the potassium phosphate concentration at pH 2.7 five times from 0.02 to 0.10 *M* (buffer 2) produced only small changes in retention, with some increase in *k* for diphenhydramine and nortriptyline being the most significant. Increasing the formic acid concentration five times from 0.02 to 0.10 *M* (buffer 4, which also results in reduction of pH from 2.7 to 2.35), gave very small changes in retention, with four analytes excluded at both buffer concentrations. Addition of 0.02 *M* sodium sulfate or sodium chloride to 0.02 *M* formic acid (buffers 5 and 6) increased retention substantially to values similar

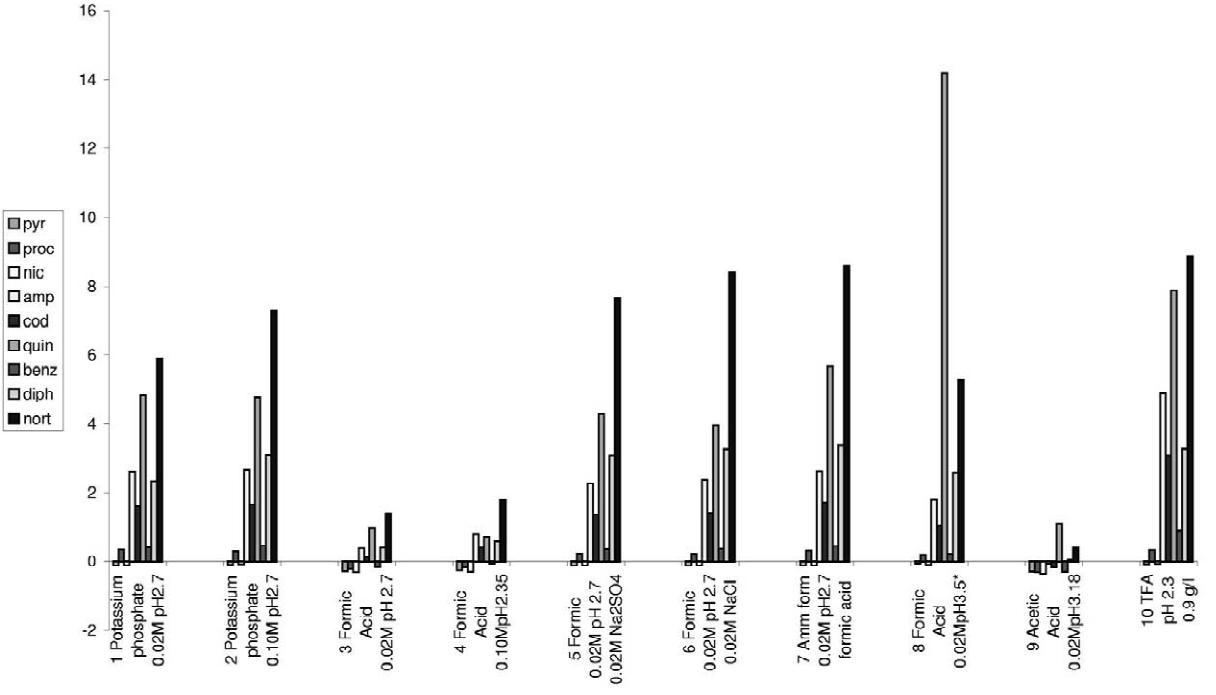

Fig. 2. Comparison of *k* on Symmetry 100 column. Mobile phases as shown using acetonitrile–buffer (10:90, v/v, except diphenhydramine and nortriptyline, 28:72, v/v). Formic = formic acid; Amm = ammonium; pyr = pyridine; proc = procainamide; nic = nicotine; amp = amphetamine; cod = codeine; quin = quinine; benz = benzylamine; diph = diphenhydramine; nort = nortriptyline.

to those found in potassium phosphate buffers. Ammonium formate buffer (0.02 *M*) adjusted to pH 2.7 with formic acid (buffer 7), gave an increase in retention comparable to that produced by the addition of the sulfate and chloride salts. Addition of a considerable concentration of formic acid (approx. 0.17 *M*) was required to achieve this pH adjustment and such increased concentrations, even of volatile buffer constituents, can adversely affect MS sensitivity [4,5]. For practical use, it might be preferable to adjust pH to, e.g. 3.0, which would require considerably less formic acid, while still probably maintaining suppression of silanol ionisation. Adjustment of 0.02 *M* formic acid with ammonium hydroxide to pH 3.5 (buffer 8) produced increased retention, but for most compounds, not to the levels shown with phosphate. The very large increase in *k* for quinine is probably due to decreased protonation of the weakly basic group (pK_a 4.3 compared with 8.5 for the strongly basic group) resulting in considerable increased hydrophobic retention of this compound. Use of 0.02 *M* acetic acid (buffer 9, pH 3.2) instead of formic acid caused even more serious exclusion effects with six out of nine test compounds giving negative *k*. Again, the pH was not adjusted to avoid introduction of extraneous substances. Note that for all the mobile phases used in Table 2, the retention of the void volume marker, uracil, remained virtually unaffected by changes in mobile phase composition.

The lack of major effect on *k* of increasing phosphate buffer strength (buffers 1 and 2) parallels results obtained by others [19,20], and could be explained by the levelling off of desolvation effects due to complete disruption of analyte solvation already at 0.02 *M* concentration. Note that using older generation silicas, which contain larger numbers of highly acidic silanols which could remain ionised at pH 2.7, workers observed *decreases* in retention as buffer strength increased. Such decreases were attributed to competitive ion-exchange interactions of buffer cations with these acidic sites on the older phases [22], which would probably have swamped the effects shown in our study. Similarly, the results for buffers 5 and 6 could suggest that chloride and sulfate, (which are commonly used as protein precipitants), might have similar disruption effects on analyte solvation to phosphate. However, introduction of these involatile additives is hardly of practical use for HPLC–MS. Furthermore, results for buffers 7 and 8 which contain NH_4^+ ions may be demonstrating solvent disrupting effects of *cations*, which are shown also in protein precipations [21]. Since the ammonium ion is volatile, use of ammonium formate instead of formic acid may be a useful way of overcoming exclusion effects. However, peak intensities with ammonium formate (as found in buffers 7 and 8) are 15–40% lower in electrospray positive mode compared with formic acid alone, indicating that MS sensitivity may be compromised [4,5]. Acetic acid is likely to behave in a similar fashion to formic acid. A similar explanation in terms of salting out effects would suggest that the higher ionic strength of the salt-containing buffers 5 and 6 is more successful in desolvating the hydrated bases in comparison with the weak acid solutions of buffers 3 and 9. Desolvation and salting out effects are directly related to the concentration of the ion [21]. Nevertheless, the absence of appreciable effects on the retention of uracil is in either case puzzling, since uracil would also be expected to be solvated and experience different exclusion effects in the different buffers.

A different explanation of the results is based on the very recent finding by Mendez et al. of *positively* charged sites on the surface of Symmetry 100 at low pH [23]. These cationic sites are attributed to residues of basic catalysts used in the production of the phase. In low ionic strength mobile phases, it is possible that full or partial *ionic* exclusion (rather than size exclusion) of similarly charged bases occurs, leading to negative or reduced *k*. These repulsion effects would be expected to decrease in mobile phases of higher ionic strength.

We are presently carrying out additional experiments to identify further the nature of these exclusion effects.

3.3. Comparison of buffers at pH 7.0

Table 3 shows a comparison of performance factors for a number of columns tested with phosphate buffers and ammonium acetate at pH 7. The

Table 3
Evaluation of columns at low pH. Mobile phases: a = acetonitrile–0.025 *M* KH_2PO_4 pH 7.0, b = acetonitrile–0.0125 *M* $(NH_4)_2PO_4$ pH 7.0, c = acetonitrile–0.025 *M* NH_4COOCH_3 pH 7.0, d = acetonitrile–0.025 *M* NH_4HCO_3 pH 7.0; all acetonitrile–buffer (30:70, v/v); flow-rate 1 ml min^{-1}; column temperature: 30 °C. All *N* values in plates/m

	Pyridine				Procainamide				Nicotine				Amphetamine				Codeine			
	k	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s
Symmetry C_{18}																				
a	0.95	22 200	5900	3.05	0.17	50 900	30 300	1.62	1.23	2940	336	6.00	0.55	11 300	1650	4.64	1.02	59 100	34 700	1.61
b	1.00	24 300	7000	2.89	0.20	49 700	29 400	1.62	1.16	5800	788	5.40	0.60	25 100	6140	3.33	1.07	62 000	38 400	1.55
c	0.95	21 700	6100	2.89	0.21	41 700	29 800	1.23	0.84	4450	552	5.61	0.58	23 800	7080	3.03	0.77	51 700	31 000	1.62
Discovery C_{18}																				
a	0.45	92 200	58 100	1.37	0.21	81 800	51 800	1.37	0.62	63 600	20 800	2.62	0.23	82 800	39 900	1.73	0.61	81 700	54 900	1.32
b	0.46	95 300	63 400	1.31	0.09	81 200	52 200	1.39	0.58	65 000	21 800	2.54	0.26	88 200	45 800	1.64	0.59	84 220	57 400	1.31
c	0.53	95 800	65 100	1.30	0.26	79 700	47 200	1.49	0.41	21 700	13 600	2.24	0.25	89 100	48 100	1.65	0.44	40 500	32 800	1.54
Eclipse EDB C_8																				
a	0.71	97 600	56 400	1.63	0.17	38 800	20 400	2.00	0.94	34 000	3130	6.45	0.29	50 400	14 300	3.38	1.01	90 500	51 500	1.81
b	0.69	106 000	63 000	1.51	0.16	41 600	21 400	1.97	0.84	41 100	5110	5.54	0.41	57 700	19 800	3.02	0.91	86 900	51 200	1.78
c	0.69	104 000	64 000	1.55	0.17	38 700	21 800	1.94	0.41	5080	1000	8.55	0.39	58 000	23 500	3.13	0.58	13 400	–	0.70
d	0.69	105 000	66 000	1.45	0.23	39 500	22 400	1.95	1.06	48 300	7060	4.48	0.55	26 800	14 700	2.49	1.23	78 200	64 300	1.21
Chromolith																				
a	0.50	92 000	36 200	2.13	0.10	72 600	14 900	3.10	1.88	2620	245	6.96	0.54	8520	1400	4.66	0.68	16 000	3470	3.95
b	0.49	97 800	42 800	2.01	0.09	89 900	17 200	3.20	1.60	3780	500	6.32	0.44	25 300	3270	4.83	0.62	18 300	4120	3.97
c	0.48	90 700	37 300	2.09	0.10	106 000	23 300	2.95	1.22	4490	600	5.91	0.34	26 100	2360	5.63	0.53	14 300	7500	2.47

	Quinine				Benzylamine				Diphenhydramine				Nortriptyline				Mean column			
	k	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s	*k*	*N*	N_{df}	A_s
Symmetry C_{18}																				
a	2.44	29 400	6470	3.68	0.26	17 900	2560	4.73	10.7	12 200	1250	6.96	14.1	5760	588	6.05	3.49	23 500	9300	4.26
b	2.91	34 300	7360	3.85	0.26	80 700	16 700	2.95	12.4	29 800	3880	6.05	18.7	19 600	2950	5.77	4.26	36 800	12 500	3.71
c	2.13	22 300	5940	3.05	0.26	39 200	16 000	2.61	8.77	23 200	3990	5.03	15.6	14 400	3020	4.72	3.35	26 900	11 500	3.31
Discovery C_{18}																				
a	1.38	65 300	30 000	2.07	0.09	75 000	33 700	1.81	5.45	61 900	21 900	2.66	6.54	55 600	18 500	2.84	1.73	73 300	36 600	1.98
b	1.39	65 300	27 400	2.15	0.12	79 000	43 800	1.53	4.97	66 000	23 500	2.76	6.46	60 900	22 100	2.76	1.66	76 100	39 700	1.93
c	1.10	70 400	37 900	1.68	0.17	77 600	48 400	1.58	3.52	75 600	36 000	2.04	6.27	68 100	33 000	2.11	1.44	68 700	40 200	1.74
Eclipse EDB C_8																				
a	2.57	43 100	7560	5.03	0.22	45 100	34 300	1.61	10.7	30 200	4120	8.36	12.5	23 120	3890	7.41	3.23	50 300	21 700	4.19
b	2.34	41 000	7950	4.96	0.20	43 300	33 700	1.69	9.71	28 600	3980	8.21	12.2	24 600	5120	6.42	3.05	52 300	23 500	3.90
c	1.70	61 900	16 800	3.75	0.21	54 400	48 200	1.41	6.75	37 100	9200	5.54	11.3	31 600	9490	4.97	2.47	44 900	24 400	3.50
d	3.10	52 200	10 800	4.36	0.24	66 200	24 800	2.92	12.9	31 300	4310	8.43	15.3	24 200	4570	6.95	3.92	52 400	24 300	3.80
Chromolith																				
a	2.48	1700	320	4.78	0.23	25 600	4330	4.30	11.4	6030	1030	4.63	14.5	5240	1040	4.43	3.59	25 600	6990	4.33
b	2.08	2120	320	5.16	0.18	66 500	13 600	3.31	9.48	9550	1190	5.81	14.0	10 400	1380	5.24	3.22	36 000	9380	4.43
c	1.60	2850	740	4.21	0.15	60 300	13 300	3.22	7.76	11 600	840	6.43	11.7	12 200	3280	3.74	2.65	36 500	9910	4.07

''semi-volatile'' ammonium phosphate buffer was also included as before. Whereas both phosphates have good buffer capacity at pH 7.0, ammonium acetate is not a buffer at pH 7.0, although it is still often used at this pH in HPLC–MS. Reproducibility of retention time may not always be such a critical factor when peak identification is based on mass spectra rather than on retention time alone when using relatively non-definitive UV measurements. For one column (Eclipse EDB C_8) we also used an ammonium hydrogen carbonate buffer made by adjusting ammonium hydrogen carbonate to pH 7.0 with acetic acid. Since the pK_a of hydrogen carbonate is about 6.1, it is likely that such a system could have a reasonable buffering ability at pH 7.0, within 1 unit of its pK_a.

For the phases evaluated at both pH 2.7 and 7.0 (Symmetry 100 C_{18}, Discovery C_{18}, Chromolith) considerably worse peak shapes were obtained at pH 7.0 than pH 2.7. For Discovery plate counts at pH 7.0 (using N_{df}) were about half those recorded at acid pH whereas for the monolith, the plate count is reduced to approximately one fifth of its acid pH value. As reported previously, silanols which become ionised as the pH is raised, are likely to be responsible [11–13]. However, the particularly low efficiencies of the monolith may be due to other factors connected with the different construction of such phases [16]. *k* values for all solutes on a given phase at pH 7.0 using potassium and ammonium phosphate buffers were virtually identical on all phases studied. However, Symmetry 100 and Chromolith showed appreciable increases in plate count using ammonium phosphate, e.g. for Symmetry mean $N_{df} = 12\,500$ plates compared with $N_{df} = 9300$ using potassium phosphate. Discovery and Eclipse showed smaller advantages of use of ammonium phosphate buffer. Apparently the ammonium ion may give better masking of some ionised silanol sites than potassium. There is no evidence of a similar effect at pH 3 which can be attributed to the suppression of silanol ionisation at low pH and thus the reduced importance of silanol effects.

Ammonium acetate at pH 7 gave column efficiency somewhat similar to ammonium phosphate with mean values of N_{df} which do not differ greatly for a given phase. No particular problems were noted with reduced precision of retention in this poorly buffered mobile phase, although problems might become apparent if larger sample sizes were injected. However, peak shapes of codeine (p$K_a = 7.9$) and nicotine (p$K_a = 8.0$) were considerably worse in ammonium acetate buffers compared with ammonium phosphate on those columns which gave reasonably sharp peaks for these compounds in ammonium phosphate (Discovery and Eclipse). For example, the half height efficiency of nicotine on Eclipse using ammonium acetate is only about 5000 plates m^{-1} compared with about 40 000 plates m^{-1} in ammonium phosphate; similarly, the half-height efficiency of codeine on Eclipse is less than a fifth of its value compared with when using phosphate buffers. We measured the pH of the ammonium acetate mobile phase after addition of acetonitrile (^s_wpH = 7.4). Considering that the presence of acetonitrile may lower the pK_a of the base [18], clearly codeine (aqueous p$K_a = 8.0$) and nicotine (aqueous pK_a 7.9) may be close to the point of half-protonation in the mobile phase, where the ionisation state of the compound is very susceptible to small pH changes. As a result, variable ionisation of the compound dependent on its concentration at a particular point in the peak is possible, leading to peak broadening or distortion [23,24]. Further evidence for this hypothesis was the improvement in the peak shape for codeine and nicotine on Eclipse when the injected solutions were diluted 20 times (10 ng injected instead of 200 ng—results not shown). This improvement did not occur for other solutes whose pK_a differed more significantly from the mobile phase pH. Poor buffering ability is likely to be more serious for peaks of low *k*, which are less diluted in the mobile phase than more highly retained peaks. If broad peaks are obtained due to other reasons such as silanophilic interactions, dilution of the sample along the length of the column by such interactions may negate some of the effects of poor buffering. For example, nicotine gives very poor efficiency on Symmetry and Chromolith in ammonium phosphate buffer, but results are hardly worse using ammonium acetate. The effect of poor buffering for compounds with similar pK_a to mobile phase pH will be more serious in real samples, where mixtures of compounds are injected, or matrix compounds are pres-

ent, and/or higher concentrations of solute used compared with our investigation.

Ammonium hydrogen carbonate buffer was investigated as a substitute for ammonium acetate, and results with the Eclipse column were initially promising. Column efficiencies were generally similar for ammonium hydrogen carbonate and ammonium phosphate and the reduction in performance for codeine and nicotine was not observed, presumably because hydrogen carbonate acts as a reasonable buffer at pH 7.0. However, measurement of the pH of the buffer reservoir after 8 h of data collection using a vacuum degasser system (no gas bubbling) indicated a significant pH change to 7.2. Bubbling helium gas through a second (previously unused) portion of buffer produced a rise in pH to over 8 in a period of only 30 min. Furthermore, storage of fresh buffer overnight in a closed container at 4 °C showed gas evolution. We concluded that such buffers are unstable at pH 7.0 due to the breakdown of carbonic acid to carbon dioxide and water, and thus are clearly not suitable for use at this pH. Presumably, helium degassing removes the reaction product of the decomposition (carbon dioxide) from the mobile phase and encourages further decomposition of carbonic acid.

4. Conclusions

In RP-HPLC separations of basic solutes at low pH, semi-volatile ammonium phosphate may be substituted for conventional potassium phosphate buffers with very little change in retention or peak shape. However, substitution of formic acid (or acetic acid) at the same pH can result in substantially reduced or even negative k values for solutes. These effects could be due to size-exclusion effects of poorly desolvated cations which exist in solutions of weak acids, compared to conventional phosphate buffers. A perhaps more plausible explanation is *ion-exclusion* effects caused by positively charged sites which exist on some phases at low pH. Ammonium formate buffers give better results, although possibly at the expense of MS sensitivity. TFA is a useful volatile buffer at acid pH, giving increased retention compared with phosphate buffers due to ion pair effects. However, suppression of MS sensitivity may be a disadvantage of its use.

At neutral pH, all phases gave worse performance than at pH 3. This result is likely to be due to silanol effects, although the particularly low efficiencies obtained with the monolith may be due to other factors. Ammonium phosphate gave similar retention but improved peak shape compared with potassium phosphate, presumably due to a superior masking effect of the ammonium ion. This effect was not evident in acidic mobile phases, at least not with modern pure silica RP columns. Ammonium acetate (pH 7) gave similar retention and acceptable peak shape for some solutes. However, for solutes whose pK_a is close to the mobile phase pH, very poor efficiency was obtained, attributable to poor buffering ability of the mobile phase. These effects will be more serious in real analytical situations where higher concentrations of solute/matrix compounds are likely to be present.

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