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Effect of organic solvent modifier and nature of solute on the performance of bonded silica reversed-phase columns for the analysis of strongly basic compounds by high-performance liquid chromatography.

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Abstract

The performance of eight silica-based RP-HPLC columns was examined with nine relatively high-pK_a bases of different structure using isoeluotropic mixtures of either methanol, acetonitrile or tetrahydrofuran (THF) in combination with phosphate buffer pH 7.0. Meaningful evaluation of the columns in unbuffered methanol–water and acetonitrile–water mixtures could only be obtained for low-pK_a bases, probably due to variable ionisation effects. Buffered acetonitrile generally produced worst peak shapes for the nine higher-pK_a bases; further improvement may be gained in some cases using THF rather than methanol. Although in general high-pK_a solutes with reduced steric hindrance gave the most asymmetric peaks, the nature of the solute chosen to probe activity could give considerably different results of relative column performance when using a given mobile phase.

Keywords: Mobile phase composition; Stationary phases, LC; Organic modifiers; Basic compounds

1. Introduction

Two recent studies have reported the effect of changing the organic modifier [methanol, acetonitrile or tetrahydrofuran (THF)] on the peak shape of basic compounds in RP-HPLC. The first [1] compared the performance for 2-hexyl and 2-heptyl pyridines of 8 RP columns using unbuffered solvent–water mixtures. The second [2] utilised solvent mixtures buffered with phosphate at pH 7.0 to compare peak shape of a much wider range of pyridine derivatives of different stereochemistry and pK_a, including compounds giving more detrimental interactions, using a single RP column shown to be generally suitable for their analysis. Both studies demonstrated considerable differences in performance for some compounds depending on the choice of modifier, with acetonitrile giving significantly worse results than methanol or THF. These investigations indicated that the modifier may be a neglected but important factor in the optimisation of the chromatography of basic compounds, which still give rise to difficulties in RP-HPLC. Nevertheless, both studies utilised pyridine derivatives of related structure and relatively low pK_a; some doubt exists as to whether these conclusions are applicable to other unrelated analytes. In the present investigation, nine different analytes of unrelated structure, including compounds

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of much higher pK_a, have been analysed on eight different columns, using buffered mobile phases containing these different modifiers. Some of the analytes were chosen as they have been used previously to show differences in the performance of RP columns; most of the solutes are important pharmaceuticals, so the results of the present study should also lead to some practical recommendations for their analysis. The assessment of columns is typically performed with mixtures which include only one or two basic probes, or with compounds which have related structure. It is known that column performance as measured by asymmetry factor (A_s) and column efficiency measurements (N) may not be consistent for unrelated analytes. Thus column "A" may perform better for compound "X", while column "B" performs better for compound "Y" [2]; Snyder and co-workers claimed that ranking of RP columns according to activity may depend to some extent on the nature of the solute, although few other studies report such work [3]. Thus, the inclusion of nine different compounds has allowed a further investigation to be performed, that of the variation in performance for a given column between these different analytes, and the relative degree of difficulty of their analysis which might be related to features of the compound such as pK_a and stereochemistry. It is also of interest to assess how consistent degree of difficulty is from column to column and whether performance can be reliably assessed with one or two significant basic probes. Comparison of RP columns using a larger number of basic solutes has exceptionally been reported [4,5]. However, silica-based columns were only examined with methanolic mobile phases, and the analytes studied were in-house pharmaceuticals which are not readily available.

We have again chosen to use organic solvent–pH 7.0 phosphate buffer for this study. Many authors (see, e.g., [6]) report that peak shapes are generally worst at this pH. Around pH 7, support silanol groups and basic solutes are often partially ionised. Therefore, this pH often presents the greatest challenge in obtaining good peak shape and high column efficiency. At lower pH, peak shape may be improved due to reduced dissociation of silanols, whereas at higher pH, decreasing protonation of the base may improve peak symmetry. In each case, reduced ion-exchange interaction should result. Nevertheless, Bidlingmeyer et al. [7] showed that an increase in accessible silanol concentration could lead to improved peak shapes for basic compounds, possibly due to reduced silanol overloading effects. Others [4,5] suggest that the optimum pH may depend on the individual analyte. Thus, additional studies at different pH values are necessary to complement the present study, since relative column performance may vary with pH.

Some initial assessment of the columns using unbuffered mobile phases was carried out. Some reports advocate testing in this way [8], although others claim that analysis of ionogenic solutes in unbuffered solutions gives rise to irreproducible results or that these conditions are unrepresentative, being remote from usual practice [2,6,9]. Thus, it was hoped that the present study might shed more light on the advisability of column testing in unbuffered mobile phases.

2. Experimental

The HPLC system consisted of P200 pump, UV 100 detector (time constant 0.05 s, 5-μl flow cell) operated at 254 or 215 nm (Thermo Separation Products, San Jose, CA, USA) and 7725 valve injector with 2-μl loop (Rheodyne, Cotati, CA, USA). We attempted to keep the dead volume of the system to a minimum, and used relatively large diameter columns to limit extra-column effects. These precautions were necessary due to the rather low k' of some analytes in the solvent systems utilised. N was determined from peak widths at half height ($w_{0.5}$) using the formula $N=5.54(t_R/w_{0.5})^2$. 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; both measurements were made using a Model 2000 data station (Trivector, Bedford, UK). All results were the mean of at least duplicate injections. The columns used (all 5 μm particle size, 25×0.46 cm I.D.) were Inertsil ODS (surface area 350 $m^2 g^{-1}$, carbon loading 18.5%, pore diameter 15 nm) Inertsil ODS-2, (surface area 320 $m^2 g^{-1}$, carbon loading 15%, pore diameter 10 nm) Inertsil ODS-3 (surface area 450 $m^2 g^{-1}$, carbon loading 15%, pore diameter 10 nm) all from GL Sciences (Tokyo, Japan); Symmetry C_{18} (surface area 330 $m^2 g^{-1}$, carbon

loading 19.5%, pore diameter 9 nm) from Waters (Milford, MA, USA); Kromasil C_8 (surface area 340 $m^2 g^{-1}$, carbon loading 12%, pore diameter 10 nm) and Kromasil C_{18} (surface area 340 $m^2 g^{-1}$, carbon loading 19%, pore diameter 10 nm) both from Anachem (Luton, UK); Supelcosil ABZ Plus (surface area 170 $m^2 g^{-1}$, carbon loading 12%, pore diameter 10 nm) from Supelco (Bellefonte, CA, USA) and Purospher C_{18} (25×0.4 cm I.D., surface area 500 $m^2 g^{-1}$, carbon loading 18.5%, pore diameter 8 nm) from Merck (Darmstadt, Germany). All columns were operated using a flow-rate of 1.0 $cm^3 min^{-1}$ apart from the Purospher column (0.9 $cm^3 min^{-1}$). All analyses were performed at 30°C with the column thermostatted in a block heater (Model 7980, Jones Chromatography, Hengoed, UK). Buffers were prepared by dissolving the appropriate quantity of KH_2PO_4 in pure water, and adjusting the pH with KOH solution of the same molar concentration, in order to maintain $[K^+]$ constant. Buffer pH was measured before addition of the organic modifier. Injection of uracil using a mobile phase of acetonitrile–water (40:60, v/v) was used to estimate column void volume. All analytes were obtained from Sigma-Aldrich (Poole, UK) except 2-[N-methyl-N-(2-pyridyl)-amino]ethanol (PAE) which was a gift from SmithKline Beecham Pharmaceuticals (Tonbridge, UK). Each solute was made up at a concentration of 100 $mg l^{-1}$ in the relevant mobile phase. The new columns were tested first with unbuffered eluents; at least 100 column volumes were purged through before use with each new mobile phase.

3. Results and discussion

The columns were selected from the newer generation of RP materials which are, according to manufacturers' data, prepared from very pure silicas and are generally recommended for the analysis of basic compounds. The Supelco column is an electrostatically shielded RP containing ion-exchange groups intended to repel basic analytes from the surface. Each column was tested initially using the modified form of the Engelhardt procedure [8,10]. Approximately isoeluotropic unbuffered methanol–water and acetonitrile–water mixtures were used as the mobile phase. Table 1 shows excellent results for the neutral probe benzene, with some columns yielding almost 100 000 plates m^{-1}. Good results were also obtained with phenol, indicating that these columns may also be suitable for analysis of acidic solutes. All columns eluted aniline before phenol

Table 1
Effect of organic modifier on column performance of solutes with unbuffered mobile phases

Column	Pyridine			Aniline			Phenol			Benzene		
	k'	N	A_s	k'	N	A_s	k'	N	A_s	k'	N	A_s
Inertsil ODS	0.87[a]	13 700	1.65	1.10	18 400	1.09	1.52	17 100	0.99	5.47	18 600	0.95
	0.77[b]	17 500	1.77	1.77	21 100	1.07	1.86	17 000	1.43	7.76	23 300	1.02
Inertsil ODS-2	0.69	2 700	2.35	0.87	7 600	1.18	1.27	19 300	1.19	5.63	24 100	1.21
	0.58	2 480	1.82	1.29	10 700	1.24	1.37	16 000	1.22	6.34	20 500	1.07
Inertsil ODS-3	1.00	8 670	1.74	1.26	12 500	1.23	1.75	16 000	1.10	8.43	17 300	1.71
	0.77	16 300	1.47	1.93	19 300	1.06	1.94	18 400	1.27	9.31	20 800	1.06
Kromasil C_{18}	1.13	1 170	4.70	1.13	17 600	1.34	1.58	17 200	1.09	7.00	19 100	1.03
	1.18	520	8.36	1.74	21 100	1.41	1.75	23 800	1.13	8.53	19 300	1.06
Kromasil C_8	1.04	1 170	5.33	1.04	20 700	1.19	1.48	20 200	1.11	4.49	22 500	1.03
	1.00	680	6.55	1.66	23 000	1.12	1.78	22 000	1.11	6.57	19 000	1.05
Symmetry C_{18}	1.39	2 600	2.90	1.50	16 800	1.18	1.87	16 100	1.09	7.91	19 500	1.03
	1.18	6 250	2.48	2.09	22 700	1.08	1.92	21 300	1.06	9.42	20 300	1.00
Supelco ABZ+	0.52	12 400	1.70	0.86	15 800	1.43	1.44	13 500	1.44	3.23	16 700	1.35
	0.53	16 700	1.61	1.25	20 200	1.35	1.73	16 000	1.65	4.31	18 500	1.16
Purospher	1.26	9 860	1.63	1.03	14 800	1.32	1.21	14 300	1.24	5.27	20 400	1.18
	1.19	17 200	1.33	1.42	21 600	1.18	1.38	20 300	1.19	5.64	23 900	1.02

[a] Mobile phase methanol–water (55:45, v/v).
[b] Mobile phase acetonitrile–water (40:60, v/v). For other conditions, see Section 2.

Table 2
Effect of organic modifier on column performance for nine basic solutes with buffered mobile phases

Column	Pyridine			Nicotine			Amphetamine			Codeine			Diphenhydramine		
	k'	N	A_s	k'	N	A_s	k'	N	A_s	k'	N	A_s	k'	N	A_s
Inertsil ODS	0.59[a]	15 600	1.25	1.34	10 100	1.82	1.05	6 760	3.29	1.65	10 300	1.24	12.3	4 230	3.38
	0.71[b]	17 700	1.04	0.73	11 400	1.77	0.53	8 320	4.74	0.85	10 000	1.52	9.48	10 000	3.09
	0.74[c]	19 300	1.35	1.14	8 540	4.15	0.44	6 660	4.64	0.94	12 300	2.46	9.60	3 240	7.62
Inertsil ODS-2	0.49	5 960	2.15	0.92	6 220	2.96	0.67	2 210	5.60	1.03	9 990	1.27	7.32	9 990	3.79
	0.54	12 300	1.64	0.53	4 390	3.35	0.71	6 780	4.07	0.64	9 040	1.89	6.61	12 500	2.21
	0.61	8 490	2.17	0.90	810	4.88	0.35	2 260	5.66	0.57	8 600	2.51	4.71	4 010	4.04
Inertsil ODS-3	0.68	10 600	1.59	1.33	8 870	2.01	0.78	6 000	3.44	1.63	9 430	1.22	10.7	10 300	2.44
	0.70	15 500	1.31	0.60	9 810	1.63	0.23	5 190	2.86	0.71	7 720	1.61	7.35	12 100	1.65
	0.74	16 500	1.51	0.92	11 400	2.20	0.21	7 980	1.87	0.78	11 500	1.57	5.17	10 100	3.06
Kromasil C_{18}	0.73	2 680	3.67	1.39	390	6.21	1.86	50	10.0	1.40	6 740	2.29	13.8	100	10.0
	0.69	7 650	2.65	0.60	2 720	5.28	0.33	2 430	5.45	0.66	9 300	1.74	7.08	5 960	3.49
	0.97	1 410	6.26	[d] –	–	–	1.08	30	6.50	0.76	4 700	4.28	10.6	260	10.0
Kromasil C_8	0.65	4 260	3.35	1.14	2 240	5.20	0.88	1 610	5.72	1.23	10 900	1.96	8.43	4 440	3.30
	0.70	10 800	2.22	0.69	2 440	5.00	0.63	6 190	4.73	0.82	10 400	1.96	7.38	11 800	2.03
	0.85	2 230	5.24	1.44	120	4.84	0.44	840	6.71	0.75	10 100	3.63	6.71	3 010	4.61
Symmetry C_{18}	0.99	4 980	2.23	1.77	2 060	4.49	1.31	3 150	4.17	1.75	7 420	1.51	11.4	9 300	2.09
	1.02	10 100	1.51	1.22	440	4.93	1.51	4 880	3.83	1.15	6 530	1.99	11.0	8 810	2.45
	1.21	6 260	2.41	2.24	230	5.34	0.79	2 880	3.58	1.10	8 810	2.02	7.45	5 990	3.42
Supelco ABZ+	0.28	13 800	1.62	0.47	8 840	2.52	0.55	6 060	3.06	0.56	9 730	1.58	2.89	10 400	1.70
	0.42	15 100	1.54	0.35	7 240	2.58	0.34	6 960	3.46	0.45	7 020	2.43	4.08	9 170	2.20
	0.41	16 000	1.64	0.44	7 350	2.97	0.41	5 660	3.87	0.41	8 790	2.67	2.17	10 500	2.28
Purospher	0.77	10 200	1.56	2.30	2 780	4.19	1.79	3 120	4.19	2.82	2 480	2.59	15.3	4 870	3.69
	0.66	14 200	1.39	0.77	2 920	2.85	0.40	5 760	2.40	1.13	3 150	2.87	6.79	7 260	2.66
	1.16	17 400	1.35	4.38	1 940	5.12	0.99	2 660	4.84	2.90	1 740	4.05	11.4	6 770	3.82
Mean solute		8 510	2.18		5 190	3.68		3 620	4.93		8 370	1.71		6 700	3.80
		12 900	1.66		5 170	3.42		5 800	3.94		7 900	2.00		9 700	2.47
		10 900	2.74		3 800	4.94		3 620	4.71		8 320	2.90		5 490	4.86

[a] Mobile phase methanol–0.064 *M* phosphate buffer pH 7.0 (65:35, v/v).
[b] Mobile phase THF–0.03 *M* phosphate buffer pH 7.0 (25:75, v/v).
[c] Mobile phase acetonitrile–0.0375 *M* phosphate buffer pH 7.0 (40:60, v/v). For other details, see Section 2.
[d] Peak not eluted, assigned $N=0$, $A_s=10$ in calculation of means.

using the methanolic mobile phase, and gave a value of <1.3 for the ratio of the A_s for aniline/phenol. Furthermore, using methanol–water, all columns gave virtual coelution (ratio of $k'<1.3$) of the isomeric *o*-, *m*- and *p*-toluidines (results not shown). According to Engelhardt [8], these columns would be classified as good for the analysis of basic compounds. However, in accord with previous results [10], the analytes do not seem to reveal significant differences in the performance of these relatively inert columns. Inclusion of pyridine in the test however, does indicate significant variation, with some columns yielding extremely asymmetric peaks with low column efficiency. Good results were obtained for pyridine with Inertsil ODS as reported previously [10,12]; the slightly higher efficiency for this solute and make of column reported in the present study may be attributed to the use of higher column temperature and improved instrumentation as well as some intercolumn variation. Column efficiency measurements were based on the half-height method; for severely tailing peaks this procedure can lead to considerable inaccuracies [13]. Plate numbers calculated in this way are only definitive for peaks with A_s close to 1.0. Thus, peak asymmetry values are usually a better measure of column performance when tailing peaks are involved. Alternative procedures for measurement of N for tailing peaks are

Table 2 (*Continued*)

Column	Nortriptyline			Procainamide			Quinine			PAE			Mean Column	
	k'	N	A_s	k'	N	A_s	k'	N	A_s	k'	N	A_s	N	A_s
Inertsil ODS	13.7	1 950	5.02	0.56	9 280	1.50	7.18	7 640	2.39	0.79	14 200	1.07	8 900	2.33
	10.1	3 450	4.53	0.11	9 160	1.52	5.19	7 130	2.90	1.26	15 100	0.96	10 300	2.45
	9.98	1 820	6.61	0.17	7 750	1.46	2.52	2 650	6.88	0.78	20 200	1.12	9 200	4.03
Inertsil ODS-2	6.14	3 830	5.32	0.33	8 040	1.66	4.10	9 850	2.14	0.58	12 400	1.28	7 610	2.91
	10.1	7 200	3.12	0.19	8 140	1.45	3.34	11 500	2.27	0.94	14 600	1.26	9 610	2.36
	4.92	2 230	5.05	0.12	5 600	2.28	1.34	3 160	5.50	0.59	14 500	1.38	5 520	3.72
Inertsil ODS-3	8.15	5 330	3.71	0.50	7 540	1.55	7.41	5 550	3.44	0.83	12 200	1.21	8 420	2.29
	5.68	4 210	2.84	0.00	5 590	1.57	4.38	7 320	2.33	1.23	13 200	1.17	8 960	1.89
	3.93	4 400	3.46	0.05	8 830	1.67	1.77	3 390	3.62	0.77	17 000	1.27	10 100	2.25
Kromasil C_{18}	14.9	100	10.0	0.43	7 120	2.73	5.80	3 270	4.29	0.74	12 500	1.72	3 660	5.66
	6.03	2 830	4.15	0.03	9 060	1.48	3.77	9 180	1.82	1.17	13 400	1.27	6 950	3.04
	10.2	140	10.0	0.08	8 440	2.75	1.80	350	7.45	0.77	6 900	4.15	2 470	6.82
Kromasil C_8	7.27	3 850	3.33	0.43	8 860	2.14	5.27	1 710	4.27	0.65	16 200	1.36	6 010	3.40
	8.77	7 070	3.37	0.16	10 700	1.50	4.02	10 900	1.86	1.22	16 700	1.20	9 670	2.65
	5.43	3 440	5.01	0.14	6 210	2.39	1.59	2 360	6.76	0.69	17 700	1.74	5 110	4.55
Symmetry C_{18}	10.5	5 140	2.90	0.73	6 260	2.03	5.91	7 490	1.72	1.04	12 300	1.21	6 460	2.48
	19.4	6 380	2.57	0.59	6 250	1.70	5.19	8 420	1.69	1.49	11 500	1.13	7 030	2.42
	8.04	3 630	3.62	0.44	10 800	2.00	2.05	3 310	3.83	1.06	17 200	1.32	6 570	3.06
Supelco ABZ+	3.33	7 560	2.08	0.24	9 390	2.07	2.10	7 930	1.66	0.35	14 000	1.52	9 750	1.98
	5.39	5 230	3.01	0.05	8 250	1.83	2.44	6 900	2.33	0.74	13 800	1.43	8 850	2.31
	3.62	5 360	3.04	0.04	9 850	2.06	1.28	5 160	2.98	0.48	17 400	1.49	9 560	2.56
Purospher	19.2	3 740	3.57	0.91	2 720	2.89	14.3	700	4.53	0.66	10 600	1.34	4 580	3.17
	8.94	3 180	3.93	0.10	4 380	1.93	5.05	3 890	2.48	0.98	11 800	1.27	6 280	2.42
	12.7	3 150	4.55	0.49	4 020	3.23	8.22	380	5.10	0.89	16 700	1.25	6 080	3.70
Mean solute		3 940	4.49		7 400	2.07		5 520	3.06		13 050	1.34		
		4 940	3.44		7 690	1.62		8 160	2.21		13 800	1.21		
		3 020	5.17		7 690	2.23		2 600	5.27		16 000	1.72		

available but not widely utilised, and may under some circumstances suffer from other difficulties. We found the half-height method yielded reproducible values [13] and report these as a guide to the presence of distorted peaks, which may sometimes give misleading values of A_s (compare for example N and A_s for pyridine using Inertsil ODS-2 with buffered and unbuffered acetonitrile, Table 1 and Table 2). With unbuffered methanol–water or acetonitrile–water, performance for a given column is broadly similar, although some (e.g., Inertsil ODS-3 and Purospher) show marked improvement for all the solutes (basic, neutral and acidic) using acetonitrile. We did not test the columns in THF–water [25:75 (v/v), an approximately isoeluotropic mobile phase for pyridine] since we found irreproducible results for pyridine in this solvent, with highly distorted peaks sometimes resulting. As shown previously [2], the protonation of pyridine is less suppressed in THF–water (25:75, v/v), presumably due to the lower concentration of organic solvent. We believe this distortion results from variable ionisation of the analyte in the mobile phase [11]. To investigate the phenomenon further, we tested columns using PAE, a compound with higher pK_a than pyridine (Fig. 1), in unbuffered acetonitrile–water eluents (results not shown). PAE generated distorted peaks on most columns, however, merely buffering the mobile phase at pH 7.0 generally removed these difficulties (Fig. 2). Thus, selection of test compounds appears restricted to those which have sufficiently low pK_a so that they are not ionised in the mobile phase, but still are sufficiently difficult to analyse to reveal differences between columns. These restrictions seem to place a rather severe limitation on the testing of columns in unbuffered mobile phases.

Table 2 shows k', N and A_s for nine basic analytes on the same eight columns using approximately isoeluotropic eluents containing the three different modifiers in combination with pH 7.0 phosphate buffer; the differences in organic solvent concentration seem more exaggerated for these basic solutes than is normally found. Peak shapes may be affected by change of k' brought about by adjustment of organic solvent strength for a particular modifier [2,11]. However, we did not adjust solvent strength to give constant k' for each analyte in a particular mobile phase, due to possible changes in column properties and solute ionisation which could also result. At least k' was reasonably constant from column to column in a given mobile phase, although the electrostatically shielded phase showed significantly lower retention of most solutes. We found that compounds eluting on the tail of a previous very asymmetric peak could show improved peak shape, presumably due to some deactivation effect caused by the preceding solute. These effects could be still measured on peaks which were very clearly resolved (R_s>5). Thus test mixtures containing groups of related basic compounds, used by some manufacturers to test columns, may give an unduly favourable indication of performance. Repeated rapid injection of the same solute sometimes produced similar "loading" effects, resulting in a temporary improvement in performance. For these reasons most analytes were injected singly, rather than in mixtures with other compounds. Especially for compounds with low k', a period of time was left between injections to allow for elimination of all traces of solute from a previous injection, thus avoiding "loading effects". These precautions were observed for all experiments in Table 2, and measurements of N and A_s, as well as k' under these conditions, were generally very reproducible.

The pK_a of the chosen analytes ranged from 5.17 for pyridine to approximately 10.0 for amphetamine and nortriptyline, and Fig. 1 indicates a range of structural features present in these compounds. Snyder and co-workers [3] have summarised the factors which promote strong interaction between amines and a silica surface. These factors include higher pK_a and reduced steric hindrance around the nitrogen atom. Our previous studies [12] have provided direct experimental evidence of these factors, and in addition have suggested that whole molecule stereochemical effects may also influence peak shape, possibly by affecting penetration of the analyte to the column surface. Values of A_s for a given solute averaged over all 8 columns (Table 2) give some indication of the relative degree of detrimental interaction for each solute. Despite having the lowest pK_a, pyridine gives poor results on many columns (average A_s 2.18, 1.66 and 2.74 in buffered methanol, THF and acetonitrile, respectively), which may be attributed to the small size of the molecule and reduced steric hindrance around the nitrogen atom. Comparison of performance for pyridine in buffered and unbuffered acetonitrile of the same strength (Table 1 and Table 2) shows significant improvement in the buffered solvent, but by no means complete removal of asymmetry, as is found for PAE with most columns. Pyridine gives generally worse results than codeine and even procainamide, which have considerably higher pK_a, presumably due to steric effects (procainamide has two ethyl groups surrounding the most basic nitrogen [3]). Peak shape for procainamide is reasonable on most columns; the relatively symmetric peaks may also be partially due to its low k' in the solvent mixtures utilised [2], which are unlikely to be suitable for practical determination of this compound. Amphetamine gives on average the worst results, consistent with its high pK_a and the relative lack of steric hindrance in the vicinity of the basic nitrogen atom. Nortriptyline (average A_s on all columns 4.49, 3.44, 5.17 in methanol, THF and acetonitrile, respectively) and to a lesser extent, diphenhydramine (average A_s 3.80, 2.47, and 4.86, respectively) also give poor results, presumably for the same reasons. Amphetamine, nortriptyline and diphenhydramine have hydrogens or methyl groups in two of the positions around the nitrogen atom [3]. Steric factors may again explain why nicotine, which has the second lowest pK_a in the set after pyridine, gives generally poor results with only Inertsil ODS and ODS-3 able to generate peaks with A_s<2.0 for this solute. Nevertheless, rationalisation of the features of a compound which give rise to peak tailing is probably performed best with groups of related substances [2,12] rather than with the present solutes, which differ in so many ways. Furthermore, other factors, such as the metal content of the stationary phase, and the relative ability of

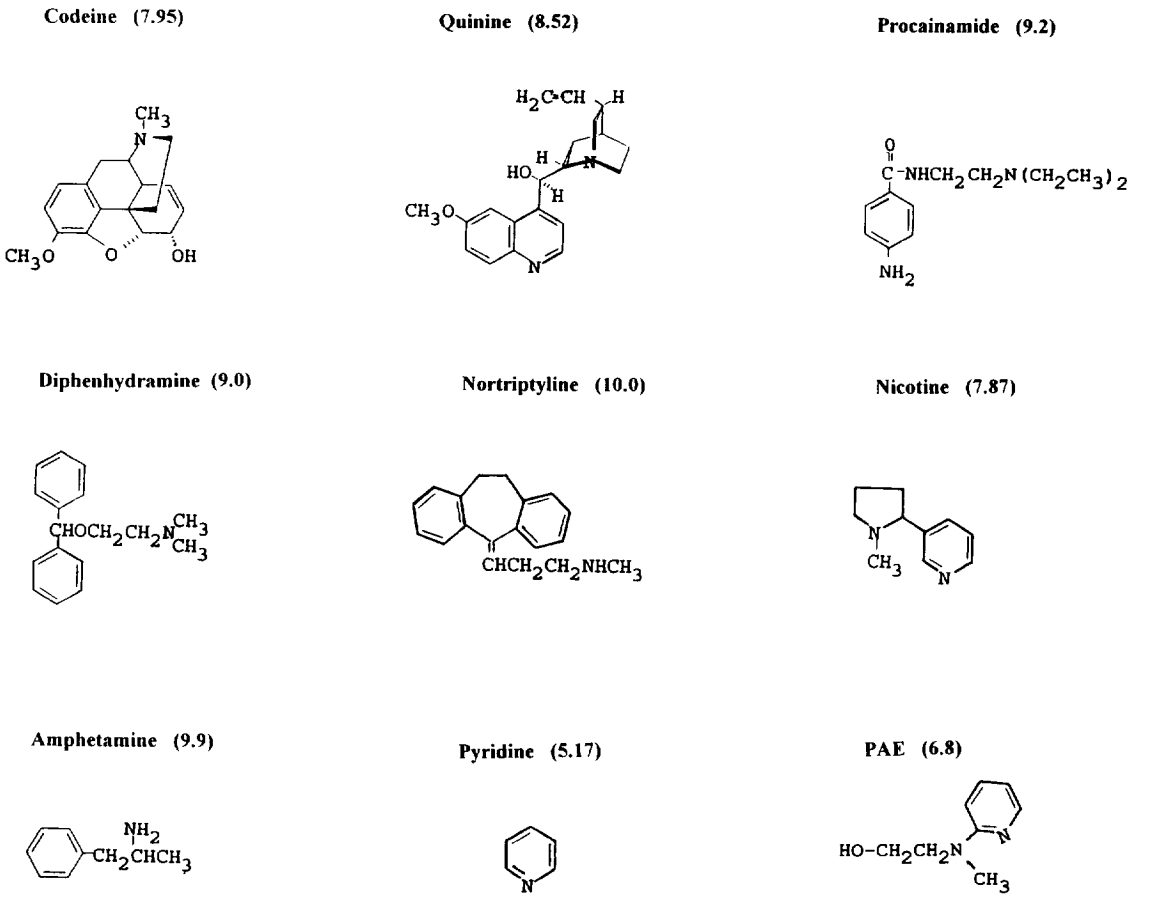

Fig. 1. Structures and pK_a [17,18] of the compounds used to evaluate columns with buffered mobile phases.

solutes to interact detrimentally with these sites, may also be important. It should be noted that the pH (see above) and modifier concentration is not necessarily optimum for analysis of any of these solutes. Thus, quinine has been shown to give generally better results when pH 3.0 buffers are utilised [10,14].

Despite these considerations for the peak shape of a given analyte averaged over all columns, it is evident that a given column may not closely reflect the average order of difficulty suggested. For example, Symmetry C_{18} gives some of the best results for the "difficult" compounds nortriptyline and diphenhydramine. However, the column seems to give poorer results for pyridine relative to some of the other columns; on average pyridine appears to be a much less demanding probe. Symmetry also gives rather poor results for nicotine. Alternatively, Inertsil ODS gives some of the best results for pyridine, PAE, nicotine, and codeine, but relatively poor results for nortriptyline. Thus, the relative performance of a column for basic solutes may depend on the nature of the analyte [3]; it may be insufficient to use 2 or 3 probes for evaluation, especially if structurally related, even if substances like nortriptyline are used, which appear on average to be challenging tests of performance. It seems necessary to record the peak shape of as wide a range of basic analytes as possible to gain meaningful overall column assessment. Most solutes chosen for this study seem to function well in revealing differences between columns. However, procainamide, which gives low k' in the solvents chosen and rather similar

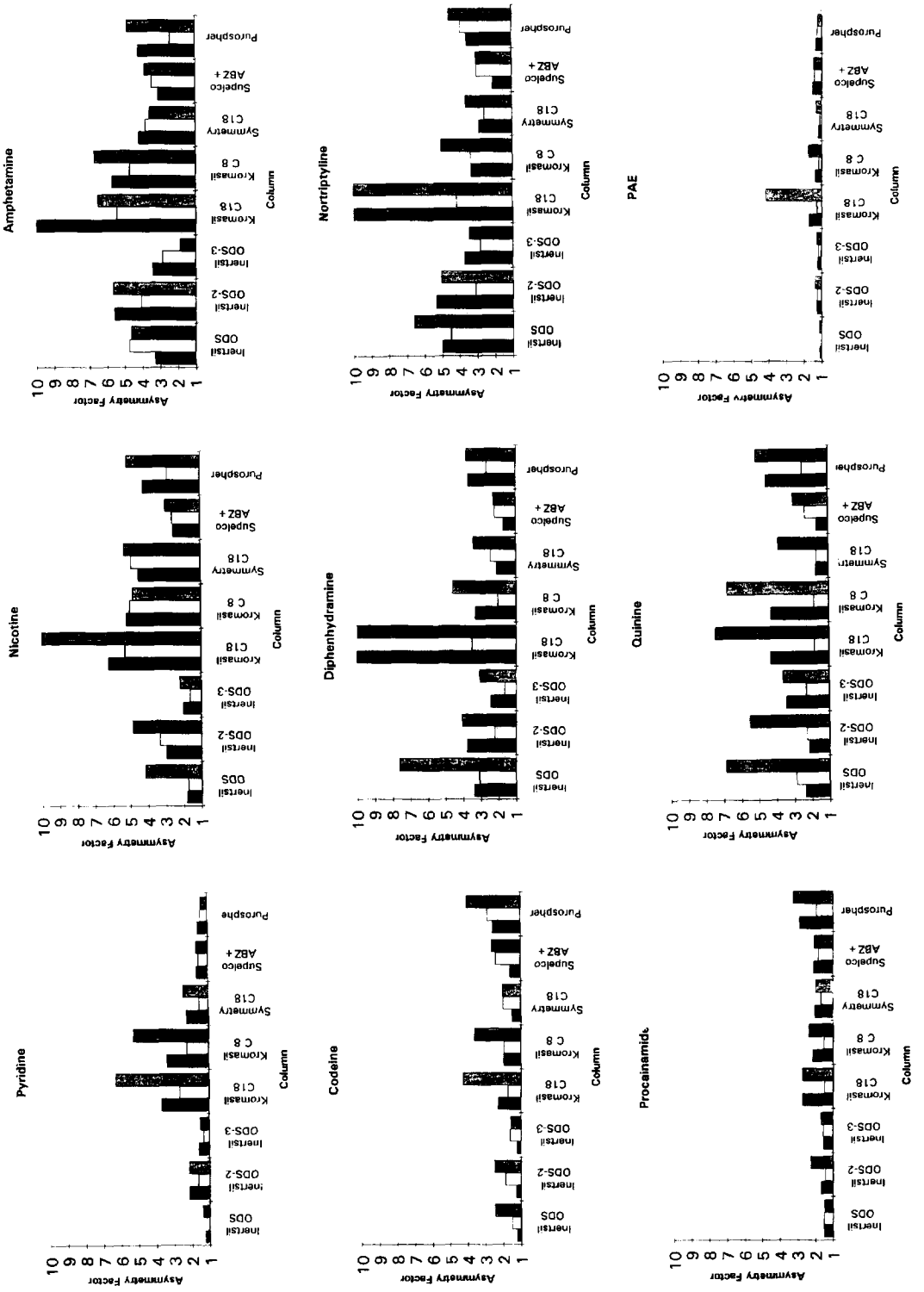

Fig. 2. Bar graphs of asymmetry factor for nine basic solutes with eight different RP columns using (black bars) methanol–0.064 *M* phosphate buffer pH 7.0 (65:35, v/v); (white bars) THF–0.03 *M* phosphate pH 7.0 (25:75, v/v); and (shaded bars) acetonitrile–0.0375 *M* phosphate (40:60, v/v). For other conditions, see Section 2.

peak shapes, and PAE, which also poses relatively few difficulties and is not widely available, seem to be less useful probes.

Differences in peak shape can occur for a given analyte and column resulting from use of the different modifiers [2]. Fig. 2 indicates clearly that for some solutes such as quinine, these differences were pronounced; worst results were obtained with acetonitrile for every column. For other solutes eg amphetamine and procainamide the differences were less marked. Amphetamine gave similar high average A_s in each mobile phase (4.93, 3.94 and 4.71 in buffered methanol, THF and acetonitrile, respectively). For less demanding compounds, like procainamide and PAE, increased analyte diffusivity in the lower viscosity acetonitrile mobile phase in accord with the Wilke–Chang equation, may make a relatively significant contribution to increased column efficiency, especially when measuring N using the half-height method [11]. Table 2 shows nevertheless, that the average A_s for all solutes (apart from amphetamine) was worst in buffered acetonitrile, although in many cases the differences are not as pronounced as for quinine. Furthermore, all solutes apart from codeine, gave improved average A_s using THF rather than methanol, although methanol and THF showed generally more similar results. Considering instead the average A_s for nine solutes on a given column, some such as Symmetry C_{18} (2.48, 2.42 and 3.06 for methanol, THF and acetonitrile, respectively), Supelco ABZ+ (1.98, 2.31, 2.56, respectively) and Inertsil ODS-3 (2.29, 1.89, 2.25, respectively) apparently show relatively little effect of changing the modifier. However, this average conceals marked differences for individual solutes. For instance Symmetry C_{18} gave considerable improvement for pyridine when using THF and for diphenhydramine using methanol; Supelco ABZ+ gave best results for nortriptyline and quinine with methanol; Inertsil ODS-3 gave much better results for diphenhydramine using THF and for amphetamine using acetonitrile. The overall performance of Inertsil ODS-3 with buffered acetonitrile is unusually good and may be related to its superior performance for acidic, basic and neutral solutes in the same unbuffered solvent. On the other hand, some columns seem to show much more pronounced variation in performance dependent on the choice of modifier. For instance, both Kromasil C_8 and C_{18} show relatively large improvement in average solute peak shape when using THF rather than methanol or acetonitrile, with THF giving better peak shape for virtually every solute. Furthermore, Inertsil ODS suffers a relatively large drop in performance for most solutes when using acetonitrile while giving similar results for methanol and THF. Obviously, optimisation of the modifier for each solute and each column should be considered. Despite the individual variations, however, in general acetonitrile gives poorer peak shapes for basic solutes when used with pH 7.0 buffer and THF may be a better choice than methanol. Factors other than solute peak shape may influence modifier choice. For instance, THF poses many practical difficulties in comparison with methanol: it is more expensive, shows reduced solubility of inorganic buffer components, has a higher UV cut-off and poses a greater hazard in use due to high flammability and formation of explosive peroxides.

Explaining why differences exist between modifiers is complex as noted previously [2]. Some differences are undoubtedly due to the large variation in organic solvent content of the isoeluotropic mixtures. However, isoeluotropic mixtures are likely to generate results of more interest to the practising analyst than comparison of mixtures containing the same concentration of organic solvent. The differences in composition could lead to different degree of ionisation of the solutes as shown previously [2,12] and in the ionisation of the buffer, which is likely to be dissociated to different extents in the different mobile phases. There are differences in the hydrogen bonding ability of the different solvents, for example, as specified in the Snyder solvent selectivity triangle. The viscosity of solvent mixtures may be the most significant factor in determining the column efficiency for less demanding compounds (see above). Furthermore, the organic solvent and its concentration can affect the solvation state of the ODS ligands and their conformation, which can influence the penetration of mobile phase components (and presumably basic analytes) to the column surface through the bonded layer and their interaction with column silanol groups [15]. Recent studies of the excess adsorption of eluent com-

ponents on RP adsorbents suggests that acetonitrile molecules interact mostly with the alkyl groups of the bonded phase with almost no influence on residual silanol groups [16]. With methanol, silica support interactions, specifically of residual silanols and siloxanes, have a stronger influence. If the modifier molecules do not interact with the silanols, the silanols may be more available for interaction with solutes. The relative influence of all these factors may depend on the exact nature of column and solute.

While performance may be influenced by the solute chosen as well as the modifier, clearly some columns are generally more suitable for analysis of basic solutes than others. For example, the C_8 Kromasil column appears to give better overall performance for basic solutes than the equivalent C_{18} column manufactured from the same base silica. Although only a single result, this finding is in agreement with that of Neue et al. [6], who suggested improved performance may be due to the greater surface coverage of C_8 phases due to reduced steric hindrance and/or the less restricted access of buffer ions to the residual silanols in the case of C_8 phases. Using an overall average A_s for all basic solutes and buffered modifiers studied, the best column was Inertsil ODS-3. However, the complex differences between all 8 columns are illustrated by the observation that Inertsil ODS appears to be the best choice for the analysis of pyridine with any of the solvents; Inertsil ODS-3 and Inertsil ODS (apart from with acetonitrile) the best choices for the analysis of codeine and nicotine, and Supelco ABZ+ or Symmetry the best choices for the analysis of nortriptyline and diphenhydramine. Of course, these recommendations might change if the eluents were buffered at acid pH.

4. Conclusions

On average, the performance of some RP columns for analysis of basic solutes is clearly much better than others. However, the performance of a given column depends to some extent on the choice of solute [3]. Some solutes are more difficult to analyse than others, and the factors which govern this degree of difficulty include high pK_a and reduced steric hindrance around the basic nitrogen atom, as has been proposed previously [3]. However, reasonable performance even for solutes which are on average most difficult to analyse, is not a guarantee of success for all basic compounds. Columns should be evaluated with as large a number of unrelated basic compounds which cover a range of pK_a values and stereochemistry, as is feasible.

The present study confirms for a much wider range of solutes than studied previously, that the modifier is an important but neglected factor in the optimisation of the chromatography of basic compounds. Although some columns may have unusual characteristics, most give appreciably worse performance for most basic solutes when using acetonitrile buffered at pH 7.0. For some columns, significant improvement can be obtained when using THF rather than methanol, although in general methanol and THF seem to generate more similar results. The reason for these variations is likely to involve a complex array of factors.

Testing of columns in unbuffered mobile phases must be performed with caution. Compounds of even moderately high pK_a may generate distorted peaks in such mobile phases, attributable to variable ionisation in the mobile phase [11]. Peak distortion may sometimes be rectified simply by buffering the mobile phase. However, few compounds of low pK_a seem to present challenging tests for relatively inert RP columns. Decreased reproducibility of results may be another difficulty associated with use of unbuffered mobile phases.

Care is necessary when testing columns to avoid injection of compounds which give strong interaction with active sites in mixtures with other similar compounds. Deactivation of the column by previous solutes may generate unrealistically favourable results for later eluting compounds. Analysts should also beware "loading" effects caused by repeated rapid injections of analyte solutions. Both of these effects are potential sources of method irreproducibility and provide additional reasons for optimising columns and mobile phases to avoid asymmetric peaks in the practical determination of basic compounds.

Further studies at alternative pH values are necessary to complement the present investigation, since relative column performance may vary with pH. It

should be noted also that while none of the columns used here showed appreciable changes during the entire course of the investigation, the present study does not address their long-term stability and repeatability. Some very interesting studies have been performed in this area by Kirkland and co-workers [19].

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