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Effect of temperature and flow-rate on analysis of basic compounds in high-performance liquid chromatography using a reversed-phase column

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Abstract

The peak shape and retention of some basic probes together with a neutral reference compound were investigated as a function of temperature and flow-rate using a reversed-phase HPLC column at both pH 3.0 and pH 7.0. The retention of bases often showed an anomalous increase with temperature; retention mechanisms are complex as shown by studies of the effect of buffer cation concentration on retention. Considerable improvements in column efficiency for bases may result from operation at elevated temperature. Improvements did not seem attributable either to incidental changes in the retention factor, or (in this particular study where low sample masses were utilised) to the influence of sample load. The optimum flow-rate for highest efficiency is generally lower for basic compounds than neutrals, and due to the steepness of the Van Deemter curves obtained, high flow-rates appear to be particularly detrimental in the chromatography of basic compounds.

Keywords: Peak shape; Temperature effects; Flow-rate; Basic compounds

1. Introduction

The analysis of strong bases by HPLC using silica-based RP columns continues to be of concern to many chromatographers, especially those who are involved with the analysis of pharmaceuticals and biomedically significant compounds, many of which contain basic groups. Such compounds may generate poor peak shape leading to difficulties with quantitation and resolution or variable retention dependent on sample mass. In a series of papers, we have examined the effects of a number of factors on the chromatography of these solutes including analyte structure and basicity [1,2], pH of mobile phase and organic modifier [2–4], sample mass [5], length of bonded chain and effect of embedded polar groups [6]. Poor peak shape of basic compounds, however, involves a variety of factors [7]. It is likely that kinetic phenomena and overloading effects, both involving underivatised silanol groups on the surface of the RP are responsible; however, the relative contribution of these different effects appears to be complex.

Relatively little work has been carried out on the effects of other variables such as temperature and flow-rate on the chromatography of bases. In some recent theoretical papers [8,9] it has been suggested

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that in linear chromatography (i.e. in a non-overload situation) tailing may be due to a mixed retention mechanism combining a classical adsorption on a non-selective surface having moderate adsorption energy and fast adsorption/desorption kinetics, together with strong selective interactions with a small number of 'active' sites having high interaction energy and slow kinetics. The same conclusion was reached by other workers using a different theoretical approach [10]. For basic compounds in RP chromatography, the two sites are likely to be alkyl ligand chains and silanol groups, respectively. It was recommended that to reduce tailing, the rate coefficient of mass transfer on the active sites should be increased [9]. By its effect in increasing the slowest rate coefficient of mass transfer to a level closer to that of the 'fast' sites, the relative contribution of the active sites to tailing and retention processes may be decreased. It is possible that at high enough temperature, the effect of 'kinetically disfavoured' sites is removed completely. However, the stability of the column at elevated temperature may be an important factor [9]. A further conclusion of the study was that efforts to perform faster analyses by using higher mobile phase velocities would be likely to give increased problems with peak tailing.

More experimental data is desirable to investigate these theoretical predictions. In the present work, it was decided to explore from a practical viewpoint the effects of higher temperatures and flow-rate on the peak shape of basic compounds. Pyridine (pK_a = 5.2), quinine (8.5) and nortriptyline (10.0) were chosen as representative compounds of low, moderate and high pK_a, together with benzene as a neutral reference compound. The retention and peak shape of these compounds was investigated at both pH 3.0 and pH 7.0, which are considered the low and high pH limits for long-term stability of RP columns [7]. The column (Inertsil ODS-3) was chosen since it gives generally good performance for the selected probes without excessive tailing [3,4]. Furthermore, this phase generates rather low back pressures [7] allowing the investigation of column performance over a reasonable flow-rate range. The aim of the present paper is not to attempt a detailed theoretical analysis of the results, but to present experimental data with some brief possible rationalisation. The mechanisms involved in the separation of basic compounds are complex, and it is hoped the present paper will point the way for further studies which may more conclusively resolve some of the issues raised.

2. Experimental

The HPLC system consisted of P200 pump, UV 100 detector (1 μl flow cell) operated at 254 or 265 nm (Thermo Separation Products, San Jose, CA, USA) and 7725 valve injector with 2-μl loop (Rheodyne, Cotati, CA, USA). Connections were made with minimum lengths of 0.0127 cm I.D. tubing. These precautions were taken to minimise extra-column effects. The band spreading of the system without the column was measured by direct connection of the injector to the detector. A mixture of all four compounds (to maximise possible system adsorptive interactions) was injected using a mobile phase of acetonitrile–phosphate buffer pH 7.0 (35:65, v/v) at 1.0 ml min^{-1}. This gave a 1 σ value of about 3.5 μl, which can be shown to give a decrease in efficiency of less than 10% even for an extreme case of an unretained peak on this column with 20 000 theoretical plates [11,12]. The column used was Inertsil ODS-3V (5 μm), 25 cm×0.46 cm I.D., surface area 430 $m^2 g^{-1}$, % C=15, from GL Sciences, Tokyo, Japan. Column efficiency was determined using the Dorsey–Foley equation [13], $N_{df} = 41.7(t_r/w_{0.1})^2/(A_s + 1.25)$ which has been shown, also by others, to give a reasonable estimate of true efficiency for skewed peaks [14]. Berthod advocated the Dorsey–Foley procedure for calculation of the efficiency of skewed peaks to give meaningful Van Deemter plots [15]. In the overloading experiments, where right-angled triangle peak shapes may occur, efficiency was determined using the method described by Snyder et al. [16]. 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; all measurements were made using a model 2000 data station (Trivector, Bedford, UK). All results were the mean of at least duplicate injections. Preparation of buffers was as described previously with pH measured before addition of organic solvent [3,4]. Temperature control was achieved by immersing the column and injector in a thermostatted water

bath. A 3 m×0.5 mm I.D. length of stainless steel tubing connected between the pump and injector and also immersed in the waterbath was used to preheat the mobile phase before delivery to the column. Column void volume was measured by injection of uracil. The performance of the column used in the present study was rigorously checked at regular intervals to ascertain whether deterioration had taken place. Variations in k and N were small and of the order of a few percentages. This allowed the same column to be used throughout the study, which is very useful in comparing the results. However, for the experiment involving variation in buffer strength and effect on retention which was carried out chronologically last, a new column was substituted. This was due to some increase in k and deterioration in N and A_s for strong bases which was noted at pH 7 (but not pH 3). The performance of the new column was very similar to that of the old column. The buffer strength experiment was also repeated on the old column and gave similar results to those reported for the new column.

3. Results and discussion

3.1. Effect of temperature on retention of basic compounds

Fig. 1a (using pH 7.0 buffer) and Fig. 1b (pH 3.0 buffer) are Van't Hoff plots indicating the effect of temperature on the retention of the three bases and benzene over the range 20–60°C. The modifier concentration was approximately the same at each pH value to avoid gross differences in solute ionisation or stationary phase solvation effects, allowing some comparisons to be made. At pH 3.0 the modifier concentration was reduced slightly to increase the rather low retention factors of pyridine and quinine but still allow the elution of the considerably more retained neutral molecule (benzene) in the same isocratic run. If the Van't Hoff plot is linear and has a positive slope, the enthalpy of association of the analyte and stationary phase is constant and negative over the entire temperature range, which is 'normal' behaviour [17]. This is shown for benzene at both pH values, and reflects the reduction in retention of benzene which occurs with increasing temperature. The dependence of ln k on absolute temperature (T) is given by:

$$\ln k = -\Delta H^{\circ}/RT + \Delta S^{o}/R + \ln \phi$$

where ΔH^{o} and ΔS^{o} are the retention enthalpy and entropy, R is the gas constant and ϕ is the phase ratio. The calculated values (from Fig. 1) of $-\Delta H^{o}$ are about 2.5 kcal mol^{-1} for benzene both in 35 and 40% acetonitrile. These are of the same order as the values for benzene of 1.93 kcal mol^{-1} and 2.05

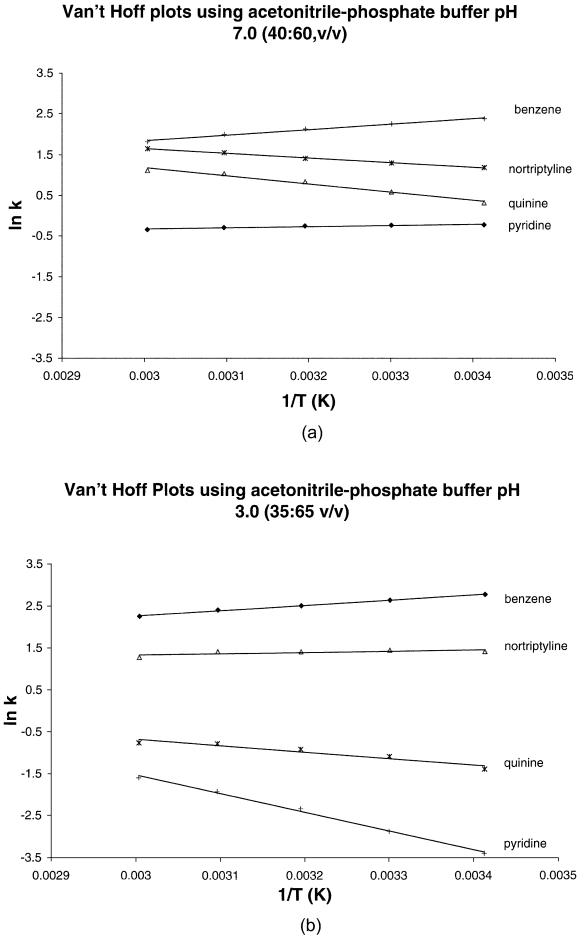

Fig. 1. Van't Hoff plots for bases and neutral reference compound using (a) acetonitrile–0.0375 M phosphate buffer, pH 7.0 (40:60, v/v) and (b) acetonitrile–0.0375 M phosphate buffer, pH 3.0 (35:65, v/v). Sample volume 2 μl, detector UV at 254 nm, column temperature 30°C. Sample mass 0.05 μg each compound except benzene 1 μg.

kcal mol^{-1} for toluene in 60% acetonitrile using a Zorbax ODS capillary reported by Chen and Horvath [18]. Pyridine shows a smaller positive slope at pH 7.0 ($-\Delta H^{o} = 0.57$ kcal mol^{-1}) but a negative slope at pH 3.0, whereas quinine shows negative slopes at both pH values. In the case of negative slopes, retention increases with temperature (anomalous behaviour). Finally, nortriptyline yields a negative slope at pH 7.0, but a line of small positive slope ($-\Delta H^{o} = 0.60$ kcal mol^{-1}) at pH 3.0. The behaviour of pyridine reflects that for N-ethylaniline (pK_a = 4.3) reported by other workers [19]. This solute gave anomalous behaviour in a pH 3.6 buffered mobile phase but not at higher pH values, where presumably this low pK_a compound is not protonated.

In an effort to shed more light on the retention mechanism operation at each pH value, the retention of the bases was investigated as a function of the buffer cation strength. A plot of k against the inverse of buffer cation concentration should give a straight line, extrapolation of which to infinite buffer concentration can allow prediction of the contribution of ion exchange to retention [20]; these plots are shown in Fig. 2. At pH 3.0, somewhat surprisingly, there seems to be little effect of [M$^+$] on retention of any of the bases. Presumably, on this high purity phase, the small number of highly acidic silanols gives a negligible contribution to retention. We have shown previously [5] that on similar high purity ODS phases, retention decreases with sample load at pH 3, which was attributed to overload of silanol groups [21]. However, based on these results, an alternative explanation of overloading may be necessary, at least on this phase (see below). The peak shapes of all three bases at pH 3.0 were reasonable (giving A_s = 1.4 or less) pointing to lower ion-exchange effects. However, peak shapes were still significantly worse than for benzene (A_s = 1.0, compare also efficiencies in Fig. 3) suggesting that small numbers of strong sites still contribute to peak shape (see next section); this result is in accord with the theoretical work of Guiochon and co-workers [8,9]. Alternatively, other factors may contribute to poor peak shape. Although retention at pH 3 seems to be mainly due to 'hydrophobic' processes, it may be more complex than for neutral molecules, involving mutual repulsion of charged species in the stationary phase [22].

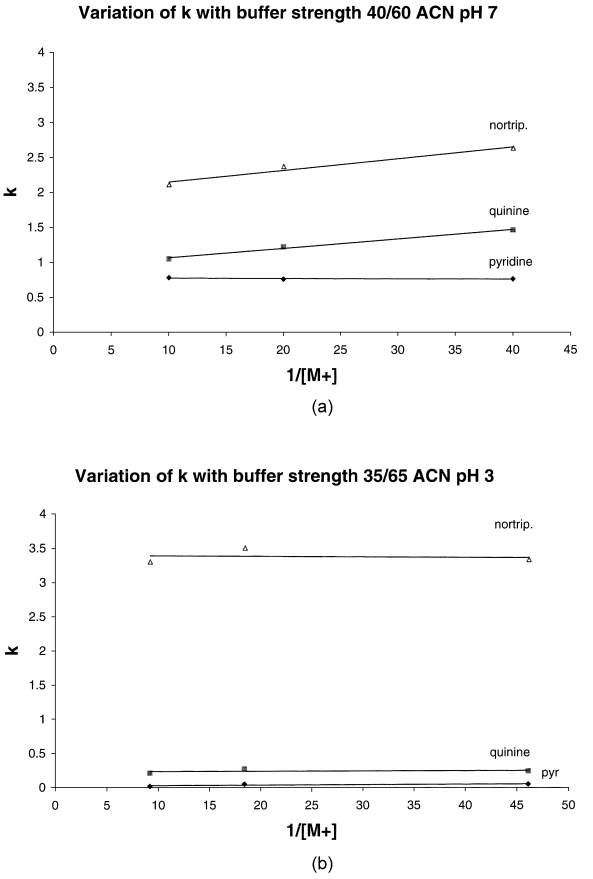

Fig. 2. Plot of k against reciprocal of buffer cation concentration. for (a) acetonitrile–0.0375 M phosphate buffer, pH 7.0 (40:60, v/v) and (b) acetonitrile–0.0375 M phosphate buffer, pH 3.0 (35:65, v/v). Other conditions as in Fig. 1.

At pH 7.0 retention of the weak base pyridine, which is mostly unprotonated, is hardly affected by buffer concentration. The retention of the stronger bases however, increases with decreasing ionic strength, which is expected considering that the average pK_a of silanol groups is about 7 [23]. Extrapolation of the lines to infinite [M$^+$] indicates there is considerable retention of even these strong bases by non-ionic processes on this phase. A contributory factor is that the data for pH and pK_a is based on aqueous values, but these are affected by the organic solvent present. The organic solvent is likely to cause a rise in the effective pH of the phosphate buffer and reduction in the pK_a of the base, leading to reduced ion-exchange and greater

hydrophobic retention [7,24]. More active silicas would be expected to give steeper plots indicative of a greater degree of ion exchange.

If hydrophobic processes are exclusively responsible for retention on this phase at pH 3, then decreased solute ionisation as the temperature is raised may provide a possible explanation of the negative slopes of the Van't Hoff plots. The pK_a of bases in water decreases as absolute temperature (T) is raised [25] according to the approximate relationship:

$$-\mathrm{d}(\mathrm{p}K_a)/\mathrm{d}T = (\mathrm{p}K_a - 0.9)/T \quad (1)$$

This equation gives good agreement with experimental data over the range 15–35°C.

The equation implies that temperature effects for strong bases are much greater than for weak bases; for instance for pyridine (pK_a 5.22 at 25°C) the drop in pK_a per degree is calculated as 0.014 (found experimentally 0.011) whereas for ethylamine (pK_a at 25°C = 10.65) it is calculated as 0.033 (found 0.032). Temperature does not seem to affect the pH of the phosphate buffer significantly [25]. It must be appreciated that these results apply to aqueous solutions and extrapolation of these results to aqueous–organic mixtures may not be fully justified. At pH 3.0 in the presence of acetonitrile, both pyridine and quinine (which has a weakly basic quinoline nitrogen) are not fully protonated. Pyridine (aqueous pK_a 5.2) is only about half protonated in 55% methanol combined with pH 3 phosphate buffer, due to the rise in effective pH of the buffer and reduction in the pK_a of the pyridinium cation caused by the organic solvent [2]. Thus, raising the temperature could cause significant reduction in the degree of protonation of pyridine (and quinine), increasing hydrophobic retention. The protonation of nortriptyline, which has a single strongly basic group is unlikely to be unaffected by temperature at pH 3.0. At pH 7, the situation is more complex since ion-exchange retention also occurs. However, on this phase, Fig. 2 indicates that hydrophobic retention is still the dominant process, even for the strong base nortriptyline. The increase in pH of the phosphate buffer and reduction in pK_a of the base due to the presence of organic solvent may lead to partial protonation of both nortriptyline and quinine at pH 7. Reduction in pK_a as temperature is raised would again lead to decreased protonation and increased hydrophobic retention explaining the negative slopes of the plot for these compounds. Alternatively, pH 7 is well above the pK_a of pyridine and temperature increases would not in this case be expected to lead to changes in solute ionisation and retention — hence the normal positive slope of the plot at pH 7.

3.2. Effect of temperature on peak shape of basic compounds

Fig. 3 shows the effect of increase in temperature from 20 to 60°C on the column efficiency of benzene and the three basic probes. The sample mass utilised for the bases was 0.05 μg. Increases in efficiency (N)

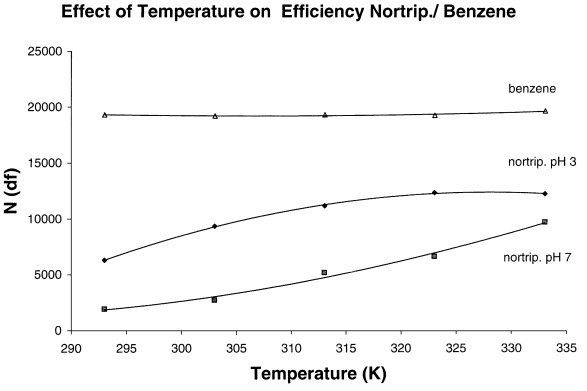

Fig. 3. Effect of temperature on column efficiency of bases and neutral reference compound. pH 7 mobile phase as in Fig. 1a. pH 3 mobile phase as Fig. 1b. Other conditions as in Fig. 1.

for neutral compounds are generally attributed to a reduction in the viscosity of the mobile phase with temperature and thus increased solute diffusivity [26]. Nevertheless, Fig. 3 shows very little change in N with temperature for benzene. Results for this neutral compound can be explained by the movement of the optimum flow-rate to higher values as temperature is raised, tending to produce a drop in efficiency at a constant flow of 1 ml min^{-1} [27]. This effect acts in the opposite direction to a small decrease in H_{min} as temperature is raised [28].

In marked contrast however, all three basic compounds show significant increases in N with increasing temperature, either at pH 3.0 or pH 7.0, although the improvement in efficiency is generally even greater at pH 7.0. For example, efficiency for quinine at pH 7 improves by a factor of about five from less than 2000 plates at 20°C to almost 11 000 plates at 60°C. At higher temperatures, the pH 3.0 and pH 7.0 plots for quinine intersect, implying that unusually for high pK_a compounds, better performance is obtained at pH 7.0 [3,4]. Similarly for nortriptyline, efficiency at pH 7 increases five-fold from less than 2000 plates at 20°C to almost 10 000 plates at 60°C. However, at pH 3.0, the efficiency for nortriptyline still doubles from about 6000 plates at low temperature to 12 000 plates at high temperature, although efficiency is invariably better for this solute at low pH. For the low pK_a compound pyridine, pH 7.0 remains preferable not only for operation at room temperature (presumably due to lack of ion-exchange effects at a pH above the solute pK_a, see above) but also at higher temperatures.

Changes in asymmetry factor (A_s) with temperature were small for pyridine at both pH values and also for benzene (data not shown). However, for quinine and nortriptyline, the large increase in N at pH 7.0 was accompanied by significant decreases in A_s for the compounds (Fig. 4). In contrast, there is relatively little variation in A_s with temperature at pH 3.0. However, although A_s is a convenient experimental measurement, it is not a fundamental chromatographic parameter and is thus on its own of less relevance than column efficiency, especially when the latter is measured using the Dorsey–Foley equation, which includes the asymmetry parameter.

These significant increases in efficiency may indeed be due to kinetic effects and the increase in

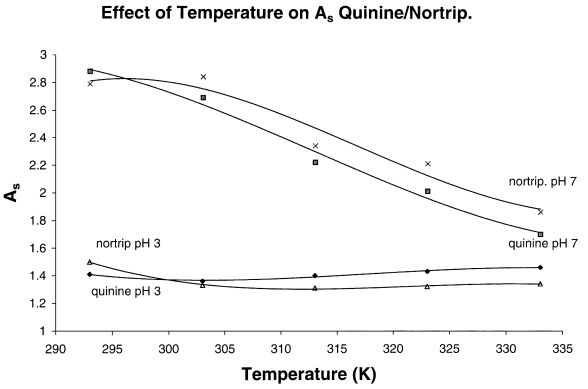

Fig. 4. Effect of temperature on asymmetry factor of (a) quinine and (b) nortriptyline. Other conditions as in Fig. 1.

the rate coefficient of mass transfer of solutes with silanols as temperature increases [9]. Increased solute diffusion coefficients in the mobile phase (improved mobile phase mass transfer) may be contributory, although similar effects might be expected for the control compound benzene. The greater improvement at pH 7.0 is likely to be due to increased silanophilic effects at pH 7.0. A large mass transfer term in the van Deemter equation would be expected to be more sensitive to changes in temperature (see also below). Changes in pK_a of bases and silanol groups with temperature (see above) may also have a beneficial effect on efficiency in reducing ion-exchange effects.

Two further effects were investigated which could influence the consequence of temperature on column efficiency. Firstly, changing the temperature changes k for the compounds, and this in itself could give rise to changes in efficiency. Secondly, it is possible that differences in peak shape with sample load (thermodynamic effects) at different temperatures might contribute to the overall result.

3.3. Effect of modifier concentration on peak shape of basic compounds

Changing the temperature from 20 to 60°C brings about changes in k; for instance, Fig. 1 shows changes in k of up to a factor of about 2 when using 40% acetonitrile–phosphate buffer pH 7.0. The C term of the van Deemter equation shows some k dependence, although the variation of H produced is

predicted to be relatively small [26]. Another possibility is surface diffusion (longitudinal diffusion within the stationary phase) which can give rise to changes in N with k [26]. It is unlikely that extra-column volumes might significantly influence values of N to a varying extent dependent on k due to the very low band spreading of the instrumentation used.

Modifier concentration was varied at constant temperature to replicate these k variations, over the range 35–50% acetonitrile at a constant temperature of 30°C. The change in modifier concentration at constant temperature produced changes in k of a factor of about 2–3, which is larger than the changes in k brought about by varying the temperature at constant modifier concentration.

For benzene, there is a small increase in efficiency as k is reduced using higher modifier concentrations. This small variation might be attributable to longitudinal diffusion in the stationary phase which increases with k or to improved solute diffusivity as the viscosity of the mobile phase is reduced [26]. However, within experimental error, no trends are discernible for bases and efficiencies seem remarkably constant with k. Caution is necessary in that although both cause changes in k, varying temperature and modifier concentration are not strictly comparable. Changes in ligand conformation occur when changing either of these variables, but these effects are not necessarily the same. Also, for bases, change in modifier concentration can bring about changes in the ionisation state for bases. However, it does appear that the large changes in N brought about by temperature variation cannot be explained merely in terms of incidental changes in k (Table 1).

Table 1
Variation in column efficiency with k brought about by changes in modifier concentration[a]

Solute	ACN (%)	k	N
Pyridine	35	0.93	16 500
	40	0.79	16 200
	45	0.64	15 900
	50	0.57	16 300
Quinine	35	2.6	2550
	40	1.9	2450
	45	1.3	2700
	50	1.1	3080
Nortriptyline	35	6.6	2960
	40	3.9	2500
	45	2.3	2960
	50	1.7	3260
Benzene	35	12.9	17 900
	40	9.5	18 700
	45	6.4	20 900
	50	5.0	21 900

[a] Mobile phase acetonitrile–phosphate buffer pH 7.0 (0.0375 M) in each case. Column temperature 30°C.

3.4. Effect of sample load on peak shape of basic compounds

Sample overload occurs much more readily for bases (at least at pH 3.0) than for neutrals. For instance, as little as 0.5 μg of base injected on to a 4.6 mm I.D. RP column may cause reduction in column efficiency, whereas amounts 50–100 greater are generally permissible with neutral compounds [5,16]. It is thus likely that in many practical situations in analytical chromatography, thermodynamic effects (loading effects) are a major contributor to poor peak shape. It is further conceivable that loading effects might vary with temperature, and thus might contribute to the differences in peak shape shown to occur with changing temperature. At pH 3.0, overloading of basic compounds generally produces right-angled triangle shaped peaks whose width at base W is given by the equation [16,26]:

$$W^2 = 16\, t_0^2(1+k)^2/N_o + 6t_0^2k^2w/w_s \qquad (2)$$

where w is the sample mass, t_0 the column dead time, k and N_o are the retention factor and plate count for a small sample mass and w_s is the saturation capacity of the column (maximum possible uptake of sample by the column). Further experimental studies at pH 3.0 have produced results in accordance with this description [5]. However, it appears the situation at pH 7.0 may be more complex, with in some cases, efficiency remaining constant or even improving with sample load over the range 0.1–20 μg injected sample. This behaviour may result from overload by protonated base of ionised silanols. A much greater number exist at the higher pH, increasing the capacity of the column. However, an alternative explanation of loading behaviour is that protonated base accumulates in the stationary phase causing mutual repulsion, which

could effectively lower w_s values [29]. This explanation seems appealing, especially at pH 3.0, in the light of the result that ion-exchange contributes little to retention on this particular column at this pH value.

Fig. 5 shows the influence of sample amount at 20 and 60°C using both the pH 3.0 and pH 7.0 mobile phases which had been used for the temperature variation experiment. At pH 7.0 it can be seen immediately that there is no significant effect on column efficiency for any of the bases until a load of at least 1 μg is utilised — this is a level at least 20 times higher than that employed in any of the other experiments. At higher loads, efficiencies for quinine show some improvement at both low and high temperature. For pyridine, some deterioration in efficiency is demonstrated for higher loads at both temperatures. These results are similar to those obtained previously on two different RP columns [5]. Results for nortriptyline above 1 μg clearly show overloading takes place more readily at 20°C than 60°C. Since k increases with increasing temperature for nortriptyline at pH 7.0 (Fig. 1), overloading effects should be higher at higher temperature according to Eq. (2). However, decrease in the protonation of nortriptyline due to pK_a changes with temperature (see above) may be important.

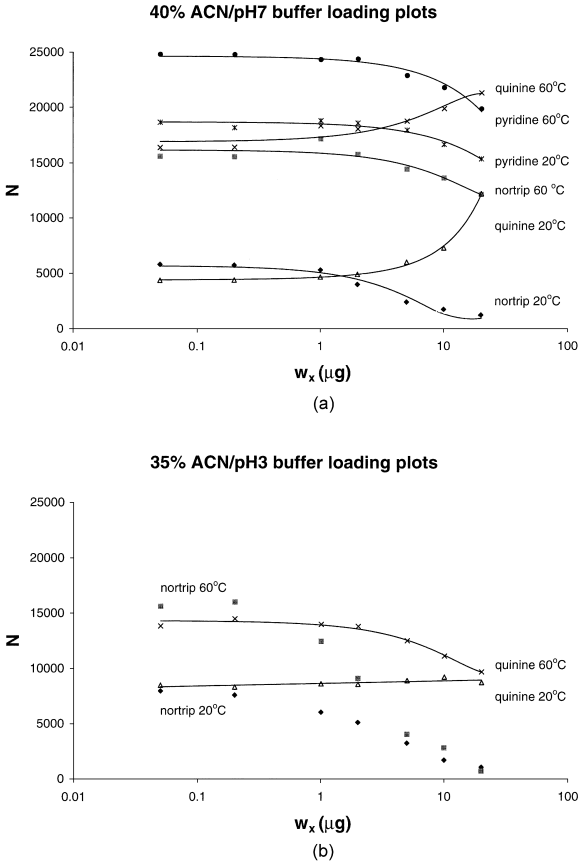

Fig. 5. Plots of column efficiency against sample load w_x at 20 and 60°C. (a) Acetonitrile–0.0375 *M* phosphate buffer, pH 7.0 (40:60, v/v) and (b) acetonitrile–0.0375 *M* phosphate buffer, pH 3.0 (35:65, v/v). Other conditions as in Fig. 1.

At pH 3.0, there is again little evidence of loading effects for quinine until well above 1 μg is injected, due to relatively high column saturation capacity for this solute measured previously [5]. The increased tendency to overload at 60 rather than 20°C may be again due to a complex effect of both change in k and pK_a of the base with temperature. For nortriptyline, results are very similar to previous findings, showing the phase more prone to overload at pH 3.0 rather than at pH 7.0. However, significant change in peak shape at either temperature does not occur until at least 0.5 μg of compound is introduced. The behaviour of pyridine at pH 3.0 was anomalous, as shown previously [5] giving fronting and splitting of peaks above 0.5 μg injected compound (results not shown). It appears this effect may be partially caused by high hydration of the pyridinium cation and exclusion effects from the column packing [30].

Sample loading effects are complex and require further study. The unusual behaviour of some compounds, e.g. quinine at pH 7.0, may be due to other factors such as buffer overload. However, it appears that no significant effect on peak shape for any of the compounds at either pH 3.0 or 7.0 results from use of sample loads up to ten times higher than the mass routinely employed in the experiments in this study (0.05 μg).

3.5. Effect of flow-rate on peak shape of basic compounds

Knox and Vasvari [31] found that the asymmetry of peaks (measured as the ratio of trailing and leading half widths) obtained on pellicular ODS

silica decreased with increasing mobile phase velocity. However, Giddings had previously predicted that kinetic tailing would be high at high velocities [32]. Fornstedt et al. [8] agreed with the predictions of Giddings, interpreting the results of Knox as being due to overloading of the pellicular materials used, which have low saturation capacities. At low velocity when the column efficiency is high the column might be overloaded, but at high velocities when the column efficiency is lower, the band is more dilute and the non-linear asymmetry reduced. This can also be seen by considering Eq. (1). When N_o is small, the first term of Eq. (1) is greater, and thus the second (overload) term has less influence on the peak width W.

In order to investigate further the effect of flow-rate on column efficiency and peak asymmetry, we studied the effect of flow-rates from 0.5 to 3.0 ml min^{-1} on performance using very small sample loads (50 ng). Consideration of the previous section indicates that such small sample loads are insufficient to produce non-linear effects.

The Van Deemter curve for the neutral compound benzene (unaffected by buffer pH) is shown in Fig. 6. It has typical shape with an optimum flow in the region of 1.5 ml min^{-1} together with increasing plate height above and below this value, attributable to mass transfer and longitudinal diffusion effects, respectively. The curves for pyridine, quinine and nortriptyline at pH 3 are considerably steeper than for benzene at higher flow-rates. These results suggest a relatively larger mass transfer term (C term) in the van Deemter equation [26] which would logically result from slow interaction kinetics of these ionised bases with ionised silanols, even at pH 3 when the number of ionised silanols is relatively small. Furthermore, a clear optimum flow-rate for any of the bases is not seen even at 0.5 ml min^{-1}. At this low flow-rate, H_{min} for pyridine and nortriptyline approaches H_{min} for benzene. Preliminary measurements indicate that the diffusion coefficients of quinine and nortriptyline in the mobile phase (D_m) are significantly lower than for benzene, which is likely to contribute to the shape of the Van Deemter curve [28]. Asymmetry measurements for the bases at pH 3 and benzene (Fig. 7) show little variation with flow.

At pH 7, the slope of the curve for pyridine is

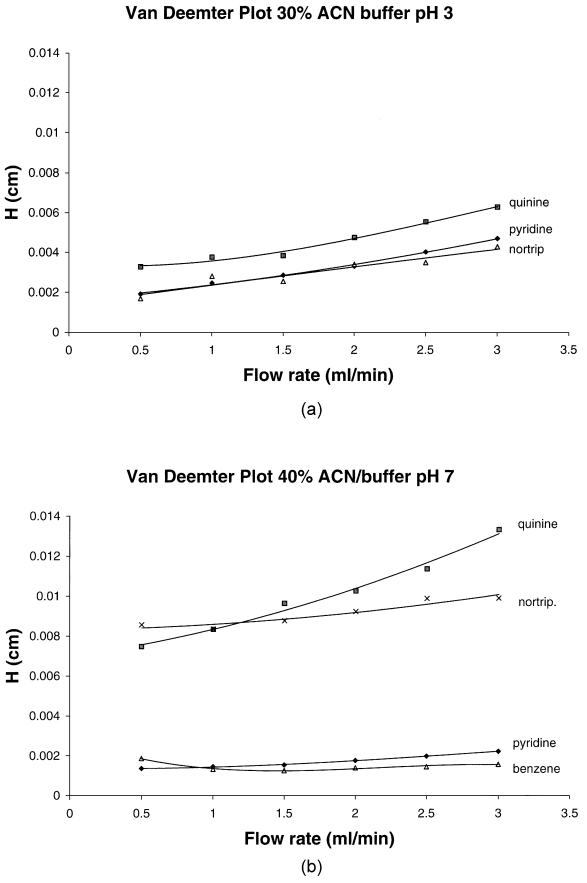

Fig. 6. Van Deemter plots of plate height (H) against flow-rate. Mobile phases (a) acetonitrile–0.0375 M phosphate buffer, pH 3.0 (30:70, v/v); (b) acetonitrile–0.0375 M phosphate buffer, pH 7.0 (35:65, v/v) Other conditions as in Fig. 1.

smaller than at pH 3 at higher flows, and is rather similar to that of benzene. The base is unprotonated at this pH which is well above its pK_a, and thus might be expected to behave similarly to a neutral compound. However, a minimum plate height is still not achieved at 0.5 ml min^{-1} in the curve. The plate heights of the stronger bases nortriptyline and quinine are generally much higher. Particularly for quinine, the slope of the curve at high flow-rate is much greater than that for benzene. Fig. 7b shows considerable decreases in the asymmetry factor for the strong bases quinine and nortriptyline with flow compared with very small changes for the (unprotonated) base pyridine. However, the asymmetry factor has no fundamental physical significance, and the

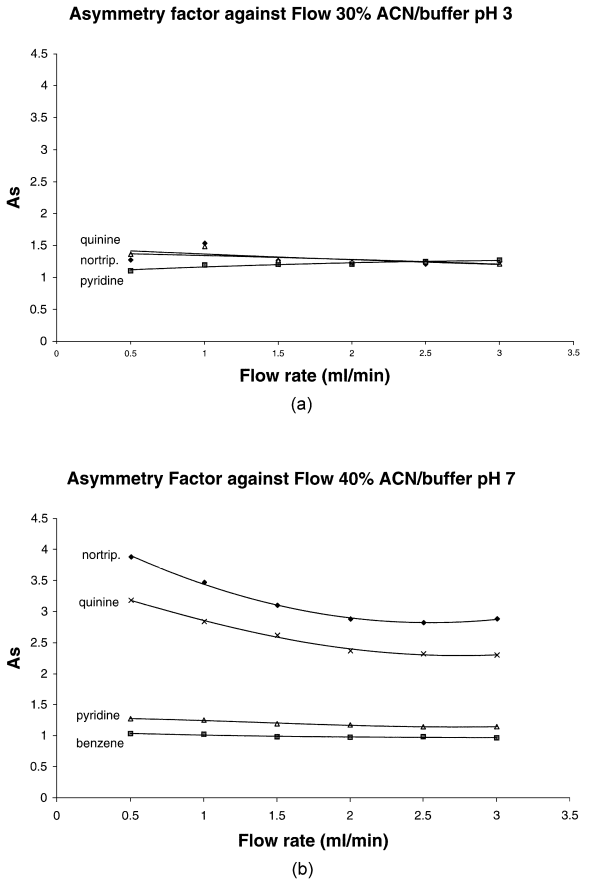

Fig. 7. Plots of asymmetry factor against flow-rate. Mobile phases and other conditions as in Fig. 6.

effect of flow on column efficiency is more consequential.

Some workers have reported van Deemter curves for neutrals at different temperatures but not for bases [27]. Further studies need to be performed in this area. Preliminary results [28] confirm that (as might be expected) at temperatures up to 60°C, much shallower curves are obtained due to improved mass transfer effects. Thus the combination of high flow-rate with high column temperature may somewhat alleviate the detrimental effects of fast flow demonstrated in the present work.

4. Conclusions

1. The retention of some basic compounds shows an anomalous increase (compared with neutral compounds) with temperature. On the pure silica RP used here, ion-exchange contributes to retention at pH 7.0, but not significantly at pH 3.0. Decrease in the pK_a of bases with temperature may be a contributory factor to anomalous retention effects.
2. In agreement with our previous studies at 30°C, considerably higher column efficiency is obtained at pH 3.0 than pH 7.0. However, there is a large increase in column efficiency (at usual flow-rates of 1 ml min^{-1}) for basic compounds with temperature, particularly at pH 7.0. The improvements in efficiency are so considerable that more thought should be given to carrying out analysis of basic compounds at elevated temperature and the development of columns which are stable at these temperatures, which may not be simple [33]. The improvement in efficiency at pH 7.0 is often accompanied by large reductions in peak asymmetry.
3. The large changes in N with temperature are not in the case of the present study attributable to variation in k brought about by temperature changes.
4. In agreement with our previous work, overloading occurs more readily at pH 3.0 than pH 7.0. However, overloading effects can show a temperature dependence for bases, which may be at least partially influenced by changes in pK_a with temperature. The very small quantities of bases (50 ng) used in this study (apart from in overloading experiments) appear unlikely to have influenced results. Thus it would appear that poor peak shapes recorded in the present study have a largely kinetic rather than thermodynamic origin.
5. The optimum flow-rate for bases can be considerably lower than neutrals. Furthermore, the steepness of the van Deemter plots obtained indicates that the use of high flow-rates is likely to be particularly detrimental in the chromatography of bases.

A summary of the results is given in Table 2. The principal findings of this study that high temperature is of considerable benefit in the chromatography of bases, whereas high flow-rate is detrimental is fully in accord with theoretical predictions [8,9].

Finally, it should be emphasised that the present results have been obtained on a single type of ODS column. More work is required on different types of

Table 2
Summary of effects of various parameters on column performance

Variable	Effect
Effect of temperature on retention	Retention usually decreases with increasing temperature for neutrals. Retention may increase with increasing temperature for bases, both at pH 3.0 and 7.0
Effect of temperature on peak shape	N increases significantly with temperature at both pH 3 and pH 7. Increase is greater at pH 7, often accompanied by considerable reduction in peak asymmetry
Effect of k (by changing modifier concentration) on peak shape	Changes in N with k (using different percentage B) much smaller than change in N with temperature, over same k range
Effect of sample load	No significant effect at loading levels used in this study (50 ng)
Effect of flow rate	Plate height increases steeply with flow for some bases. Optimum flow often considerably lower than for neutrals
Effect of buffer strength on retention	At pH 7 retention decreases with increasing buffer strength for strong bases, implying ion-exchange contribution to retention. At pH 3 retention appears independent of buffer strength.

column to confirm the universality of the results reported here.

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