Journal of Chromatography A, 1217 (2010) 276–284

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Further investigations of the effect of pressure on retention in ultra-high-pressure liquid chromatography

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ARTICLE INFO

Article history:
Received 12 August 2009
Received in revised form 6 November 2009
Accepted 13 November 2009
Available online 18 November 2009

Keywords:
Retention factors
Variation with pressure
Ultra-high-pressure LC

ABSTRACT

In this study, we investigated further the large increases in retention with pressure that we observed previously in RP-LC especially for ionised solutes. These findings were initially confirmed on a conventional silica C_{18} column, which gave extremely similar results to the hybrid C_{18} phase originally used. Large increases in retention factor of ~50% for a pressure increase of 500 bar were also shown for high MW polar but neutral solutes. However, experiments with the same bases in ionised and non-ionised forms suggest that somewhat greater pressure-induced retention increases are found for ionised solutes. Retention increases with pressure were found to be considerably smaller for a C_1 column compared with a C_{18} column; decreases in retention with increasing pressure were noted for ionised bases when using a bare silica column in the hydrophilic interaction chromatography (HILIC) mode. These observations are consistent with the partial loss of the solvation layer in RP-LC as the solute is forced into the hydrophobic environment of the stationary phase, and consequent reduction in the solute molar volume, while the water layer on the surface of a HILIC packing increases the hydration of a basic analyte. Finally, retention changes with pressure in RP-LC can also be observed at a mobile phase pH close to the solute pK_a, due to changes in pK_a with pressure. However, this effect has no influence on the results of most of our studies.

1. Introduction

The influence of pressure in LC has been considered for many years. However, this topic has attracted greater interest with the recent introduction of instruments that allow the use of inlet pressures up to 1000 bar. The main thrust of recent studies seems to be the effect of pressure on efficiency [1–5]; nevertheless, some studies have considered its influence on retention.

The change in k as a function of pressure at constant temperature can be expressed by the equation [6–8]:

$$\ln\left(\frac{k}{k_0}\right) = -\frac{\Delta V}{RT}\cdot P + \ln\left(\frac{\beta}{\beta_0}\right) \quad (1)$$

where k_0 and β_0 are the retention factor and the phase ratio under reference conditions (which are taken as atmospheric pressure), R is the gas constant, T the absolute temperature and ΔV is the change in molar volume associated with the solute's transition between the mobile and the stationary phases.

$$\Delta V = V_{stat.} - V_{mob} \quad (2)$$

Thus a graph of $\ln k$ vs P should be a straight line with slope $-\Delta V/RT$. Early studies that concentrated on the effect of pressure on retention of small neutral solutes [9–14] showed quite small increases in retention with pressure. For example, McGuffin [9,10] showed that a pressure increase from 36 to 360 bar led to an increase in retention of a homologous series of C_{10}–C_{20} derivatised fatty acids of from 9% to 24%. Ohmacht and co-workers [14] showed increases in k of 10–25% for some aromatic hydrocarbons and for some small polar compounds for an average column pressure increase of about 380 bar. However, other workers found quite large changes in retention for proteins [15–18]. For example, Chen et al. [15] showed a 3-fold increase in retention time of the enzyme lyzozyme when the average column pressure was increased from 23 to 318 bar. Liu et al. [17] found that an increase in pressure of 180 bar caused k of insulin to increase by as much as a factor of 2. Guiochon [18,19] studied the effect of pressure on the protein insulin (MW 5808) first on a conventional HPLC instrument but later at the high pressures available from a UHPLC system. An increase in retention of ~330% for a pressure increase of ~745 bar at ~25 °C was reported. Plots of $\ln k$ vs pressure were slightly con-

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doi:10.1016/j.chroma.2009.11.041

vex upward. The authors determined a general expression of the retention factor of insulin as a function of both temperature and pressure. Clearly, the increase in retention with pressure is much greater for large molecules compared with small molecules, presumably due to the larger changes in the solute molar volume when transferring from the mobile to the stationary phase. Our group previously studied the effect of pressure on the retention of a wide range of different solutes [20], including ionised acids and bases with MW < 300. Increases in retention of these ionised solutes of as much as 50% were observed for an increase in the average column pressure of 500 bar. Such large increases had not previously been reported for these relatively low MW solutes. While these large increases were found for ionised solutes, greater increases were found for polar compared with neutral non-polar solutes of similar RMM. It is likely that these larger increases in the retention are due to larger changes in the molar volume of the solvated solute as it is transferred from the mobile to the stationary phase, as a result of such solutes losing part of their hydration layer when entering the hydrophobic environment of the C_{18} layer of the RP column used in these experiments.

Tanaka and co-workers [21] demonstrated *decreases* in retention with pressure for some solutes at mobile phase pH close to the solute pK_a. This study suggested that pressure can have an effect on changing the solute pK_a. Along with most of the earlier work, it was carried out at the relatively low pressures available with commercial instrumentation available at the time. However, as indicated above, increased pressure generally results in an increase in retention in RP-LC [9–22], as long as heating effects are excluded. This condition is mostly satisfied by attaching restriction capillaries to the end of the column, as in our previous study. Nevertheless, the effects of variable frictional heating are difficult to exclude entirely, and due to the increased viscosity at higher pressure, the column will be at a slightly elevated temperature at high pressure compared with low pressure, even if the flow rate is the same and the pressure is increased by restriction capillaries. We believe that this factor contributes to the somewhat lower values of ΔV which we reported at higher flow rate (higher pressure) compared with low flow rate [20], as higher temperatures result in reduced retention, acting in the opposite sense to the effect of increased pressure.

In the present study, we have explored further some of the issues raised in our previous study in order to answer the following questions.

(1) First we wished to confirm that the unusually large changes in retention with pressure reported previously were not unique to the particular hybrid C_{18} phase used, and could be reproduced on a conventional silica C_{18} phase.
(2) For solutes of comparable molecular size, can highly polar but neutral molecules give similar increases in retention with pressure to those shown by ionised solutes?
(3) Are retention changes with pressure associated with change of solute pK_a important in the higher pressure ranges available with modern instruments?
(4) What effect does change in solute k (adjusted by varying the mobile phase composition) have on the increase in retention with pressure?
(5) Does change in the chain length of the bonded phase influence the effect of pressure on retention?
(6) What influence does changing the separation mechanism have on the effect of pressure on retention? For example, hydrophilic interaction chromatography (HILIC) appeared to be an attractive alternative mechanism to study as according to our rationalisation of the findings in RP-LC (i.e. changes in solute hydration), results should be completely different in HILIC.

2. Experimental

An ultra-high-pressure liquid chromatography system (UPLC® system) was used (Waters, Milford, MA, USA) with binary solvent manager, photodiode array (DAD) ultraviolet (UV) detection system (500 nL flow cell), and sample manager/injector valve (1 μL injections from partially filled 5 μL loop with needle overfill) was used in all experiments. Temperature was maintained at 30 °C using the UPLC oven. The columns used (all 50 mm × 2.1 mm I.D., 5 μm particle size) were: XBridge C_{18} BEH; Atlantis HILIC silica; XBridge C_1 BEH. All were custom packed by Waters (Milford, USA) in UPLC hardware designed for use with sub-2 μm packings, in order to withstand the high applied pressure. Some previous experiments using the XBridge column were repeated on a HyPurity C_{18} column, 50 mm × 2.1 mm I.D., 5 μm particle size (Thermo, Runcorn, UK) again custom packed by the manufacturer in suitable hardware. Solvents were pre-mixed and delivered from a single pump channel. Column backpressure was increased by attaching capillary tubes of 30 μm ID and lengths from 10 to 35 cm. The tubes were attached between the end of the column and the detector using a zero dead volume connector. The volume of the longest tube used was 0.18 μL, a value that is very small compared with the total extra-column volume (12.7 μL with no capillary). Therefore, the addition of tubing of this small diameter prior to the detector is not expected to affect column performance. The increases in retention that resulted from the addition of this tubing were very small, but nevertheless were corrected for in each case. With the RP column, the buffers used were 0.025 M phosphate at pH 2.8, 5.8 and 8.0, or phosphoric acid at pH 1.5. With the HILIC column, the mobile phase was ACN–water (90:10, v/v) containing 0.005 M ammonium formate adjusted to ${}_{w}{}^{w}$pH 3.0. Acetonitrile (far UV grade) and KH_2PO_4 were obtained from Fisher Scientific (Loughborough, UK). Ammonium formate was obtained from BDH (Poole, UK) and test solutes were obtained from Sigma–Aldrich (Poole, UK). The RP column was equilibrated for at least 10 h when using phosphate buffers, in order to avoid the problem of "slow equilibration", which causes the retention of ionised bases to slowly decrease and that of ionised acids to slowly increase with time [23]. Duplicate measurements of retention time were taken in each case; on average, the variation in retention time between these duplicates was about 0.15%.

3. Results and discussion

3.1. Investigation of the influence of pressure on retention using a different C_{18} stationary phase

In order to eliminate the possibility that the unusual increases in retention for ionised solutes observed [20] were due to some unusual property of the inorganic–organic hybrid phase used, we repeated some of the experiments with a conventional 5 μm particle ODS column (HyPurity C_{18}), using the same experimental conditions and with the same strong acids and bases as before as probes. The results were indistinguishable from the previous results. For example, an increase in average column pressure of 500 bar for HyPurity C_{18} caused % increases in k using acetonitrile–acidic phosphate buffer (30:70, v/v) at 0.2 mL/min of 41%, 40%, 58% and 59% for the bases propranolol, diphenhydramine, protriptyline and amitriptyline compared with 41%, 40%, 58% and 58% for XBridge. For the acids p-xylene-2-sulfonic acid and 2-naphthalenesulfonic acid the increases were 31% and 37% on HyPurity compared with 33% and 37% on XBridge. Considering that one C_{18} stationary phase was bonded to pure silica, while the other was a C_{18} based on a hybrid packing, we concluded that our previous results were not specific to the type of C_{18} phase used. In addition, the surface coverage and the retentivity of both packings

Table 1
Changes in k with pressure depending on the mobile phase composition.

Compound	% water	Average column P with no restriction capillary (bar)	Average column P range (bar)	k with no restriction capillary	Slope of ln k vs P plot	Intercept	Correlation (R^2)	% change in k for a 500 bar P increase	ΔV (cm^3/mol) at 30 °C
Amitriptyline	50%	37	37–361	0.79	0.000623	−0.253	0.994	36.5%	−15.7
	55%	37	37–375	1.24	0.000675	0.193	0.998	40.1%	−17.0
	61%	39	39–398	2.27	0.000773	0.790	0.999	47.2%	−19.5
	65%	39	39–386	4.32	0.000836	1.43	0.999	51.9%	−21.1
	70%	41	41–394	9.65	0.000895	2.23	0.999	56.4%	−22.6
	75%	41	41–422	24.9	0.000850	3.19	0.998	53.0%	−21.4
2-Ethylaniline	65%	43	43–376	3.59	0.000131	1.27	0.993	6.8%	−3.3
	70%	46	46–400	5.14	0.000156	1.63	0.999	8.1%	−3.9
	75%	46	46–391	7.76	0.000193	2.04	0.994	10.1%	−4.9
	80%	46	46–416	12.1	0.000198	2.49	0.991	10.4%	−5.0
	85%	50	50–403	19.7	0.000184	2.97	0.997	9.6%	−4.6
	90%	48	48–400	32.7	0.000175	3.48	0.982	9.1%	−4.4
3-Ethylaniline	65%	44	44–376	3.67	0.000153	1.29	0.996	8.0%	−3.9
	70%	46	46–400	5.41	0.000184	1.68	0.999	9.6%	−4.6
	75%	46	46–391	8.50	0.000200	2.13	0.996	10.5%	−5.0
	80%	46	46–419	13.8	0.000209	2.62	0.997	11.0%	−5.3
	85%	50	50–403	23.4	0.000192	3.14	0.988	10.1%	−3.9
	90%	47	47–402	39.6	0.000158	3.67	0.986	8.2%	−4.0
4-Ethylaniline	65%	49	49–376	3.52	0.000169	1.25	0.996	8.8%	−4.3
	70%	46	46–401	5.24	0.000194	1.65	0.998	10.2%	−4.9
	75%	46	46–390	8.35	0.000214	2.11	0.999	11.3%	−5.4
	80%	46	46–420	13.9	0.000220	2.62	0.995	11.6%	−5.5
	85%	50	50–404	23.9	0.000198	3.17	0.996	10.4%	−4.2
	90%	47	47–401	41.4	0.000157	3.72	0.993	8.2%	−4.0

Mobile phase aqueous component $_w{}^w$ pH 2.8 for amitriptyline, $_w{}^w$ pH 7.2 for aniline derivatives, and in admixture with ACN. Flow 0.2 mL/min.

are different. Thus the observed effects are not very sensitive to the details of the composition of the C_{18} stationary phase.

3.2. Influence of pressure in RP as a function of mobile phase composition

Our previous interpretation of the large increases in retention with pressure for polar and ionised compounds supposes that partial loss of the hydration layer on entering the hydrophobic stationary phase is involved. As such, the effect should depend on the mobile phase composition. Four compounds were tested: a strong base, amitriptyline, and three weak bases, 2-ethylaniline, 3-ethylaniline and 4-ethylaniline using ACN–phosphate buffers. Amitriptyline was tested at low pH (2.8) where it is fully ionised ($_w{}^w pK_a = 9.4$ [24]), whereas the aniline derivatives ($_w{}^w pK_a$ 4.42, 4.86 and 5.11 for 2-ethylaniline, 3-ethylaniline and 4-ethylaniline respectively [25]), were analysed under their neutral state at higher pH ($_w{}^w pH = 7.2$). We note that the addition of organic solvent separates the pK_a of the anilines and the mobile phase pH still further as the pH of the phosphate buffer (an anionic acid HA^-) is raised on adding ACN, but the pK_a of the protonated bases (cationic acids BH^+) is lowered [26]. Thus, the pK_a of these solutes was considered to be too far from the mobile phase pH to expect changes in ionisation of the solutes with pressure. The % water was varied from 50% to 75% for amitriptyline and from 65% to 90% for the aniline derivatives to give reasonable retention factors k for these solutes. The results obtained are summarised in Table 1. Plots of ln k vs pressure for all the compounds in the different mobile phases tested showed good linearity (minimum value of R^2 was 0.982, average was 0.995). All four compounds showed the same effect: an increase in the % of water in the mobile phase at first increased the pressure-induced increase in retention, followed by a decrease (see Figs. 1 and 2). For example, 2-ethylaniline gave a slope of $\sim 1.3 \times 10^{-4}$ with 65% water, which increased to $\sim 1.6 \times 10^{-4}$ with 70% water, $\sim 1.9 \times 10^{-4}$ with 75% water, $\sim 2.0 \times 10^{-4}$ with 80% water and then the slope decreased to $\sim 1.8 \times 10^{-4}$ with 85% and 90% water.

Changing the mobile phase ratio is expected to lead to modifications of the analyte solvation layer. Initially, the more water in the mobile phase, the more hydrated the analyte may become. Therefore it is reasonable to assume that there will be a greater change in the molar volume when the hydration layer is lost on entering the stationary phase. An additional factor is that it is well known that the structure of ACN–water mixtures changes dependent on its composition. There seems broad agreement from several studies that there are 3 different regions which exist. On the water rich side there is a region which extends up to a mole fraction of ACN (x) ~0.15 in which the water structure remains intact as ACN molecules are added interstitially into cavities between water molecules, without disrupting the water structure. In the range $x = 0.15$–0.75 there is a region of microheterogeneity (molecules have a preference for neighbours of the same kind), where there are clusters of molecules of the same kind surrounded by regions where molecules of the two kinds are close to each other. Finally, on the ACN-rich

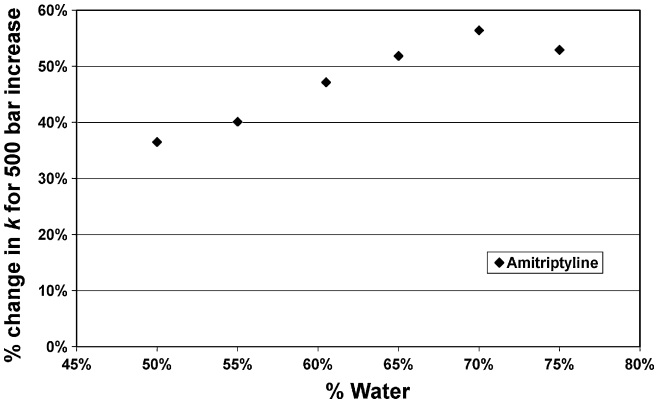

Fig. 1. Plot of % change in k for a pressure increase of 500 bar vs % water in mobile phase for amitriptyline. Conditions: XBridge C_{18} BEH, 5 μm, 50 mm × 2.1 mm, 30 °C, 1 μL injection volume, mobile phase buffered with 0.025 M KH_2PO_4 at pH 2.8, flow rate at 0.2 mL/min, UV detection at 210 nm.

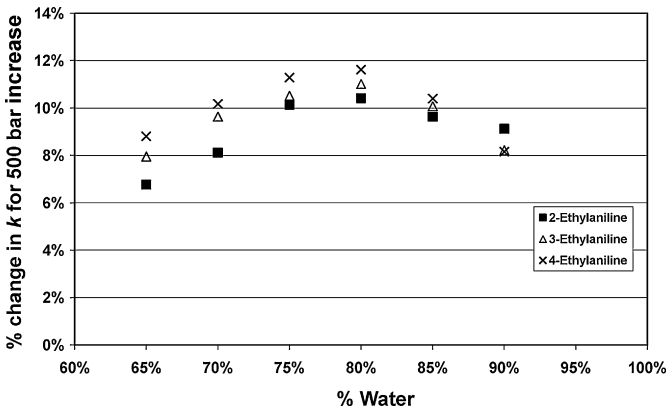

Fig. 2. Plot of % change in k for a pressure increase of 500 bar vs % water in mobile phase for 2-ethylaniline (squares), 3-ethylaniline (triangles) and 4-ethylaniline (crosses). Conditions: XBridge C_{18} BEH, mobile phase buffered with 0.025 M KH_2PO_4 at pH 7.2, flow rate at 0.2 mL/min, other conditions as Fig. 1.

side, the number of water clusters is low, and water–ACN interactions that could be discounted in the middle range now become important [27–29]. Considering that both Figs. 1 and 2 show a change of trend at 70–80% water (0.13–0.08 mole fraction ACN) it is possible that this change could be attributed to the change in the structure of the mobile phase which occurs in this region. However, there are at present no studies which detail the effect of pressure on these structural regions. Another possible contribution to the decrease in the pressure-induced increase in retention that occurs at 70–75% water may be due to viscosity effects. Using the empirical relationship of Chen and Horvath [30] at 30 °C, ACN–water (40:60, v/v) has a viscosity of 0.766 cP, whereas ACN–water (20:80, v/v) has a viscosity of 0.816 cP. While we believe the influence of frictional heating on our experiments is low due to the relatively large particle size and small column length, it is possible that some frictional heating of the column takes place resulting in some loss in retention in opposition to the gain in retention with pressure. A further addition to the effect of frictional heating is the increase in viscosity that occurs with increasing pressure. However, over the range of solvent compositions relevant to our results in Figs. 1 and 2, increases in viscosity with pressure are generally smaller as the water content of the mixture increases, which is likely to moderate this effect [31,32]. In general, these results support our hypothesis that partial loss of the solute solvation layer when entering the hydrophobic stationary phase gives rise to large increases in retention, both for ionised and for non-ionised (polar) solutes.

3.3. Influence of pressure on retention of neutral polar compounds

Previous results have shown clearly that both increasing molecular size and/or polarity give rise to larger pressure-induced increases in retention. However, we wished to investigate further whether larger polar but neutral molecules could give significant increases in retention with pressure. While proteins have been studied to some extent (see above), neutral solutes with a moderately larger molecular weight have not previously received much attention. Thus, three corticosteroids were tested: hydrocortisone (MW 362), prednisone (MW 358) and prednisolone (MW 360) using acetonitrile–water (25:75, v/v) at a constant flow rate set on the pump. As before, pressure was increased by adding 30 μm I.D. restriction capillaries of length from 10 up to 35 cm to the end of the column. Table 2 summarises the results obtained at three different flow rates. We attribute the decrease in the slope of ln k vs P with flow to greater frictional heating effects at higher flow (see above). Fig. 3 shows the increases in retention with pressure increase at

Table 2
Changes in k with pressure for three corticosteroids.

Compound	F (mL/min)	Average column P with no restriction capillary (bar)	Average column P range (bar)	Capillary length range (cm)	k with no restriction capillary	Slope of ln$k$ vs P plot	Intercept	Correlation (R^2)	% change in k for a 500 bar P increase	ΔV (cm^3/mol) at 30 °C
Hydrocortisone	0.2	33	33–507	0–35	4.799	0.000808	1.54	0.995	49.8%	−20.4
	0.3	50	50–761	0–35	4.840	0.000733	1.55	0.996	44.3%	−18.5
	0.4	67	67–849	0–30	4.858	0.000726	1.54	0.997	43.8%	−18.3
Prednisone	0.2	38	38–501	0–35	4.471	0.000820	1.47	0.995	50.7%	−20.7
	0.3	57	57–752	0–35	4.524	0.000740	1.48	0.994	44.8%	−18.7
	0.4	77	77–839	0–30	4.582	0.000745	1.47	0.999	45.1%	−18.8
Prednisolone	0.2	36	36–485	0–35	4.225	0.000850	1.42	0.991	53.0%	−21.4
	0.3	54	54–728	0–35	4.268	0.000783	1.42	0.993	47.9%	−19.7
	0.4	73	73–853	0–30	4.320	0.000779	1.41	0.999	47.6%	−19.6

Mobile phase ACN–water (25:75, v/v).

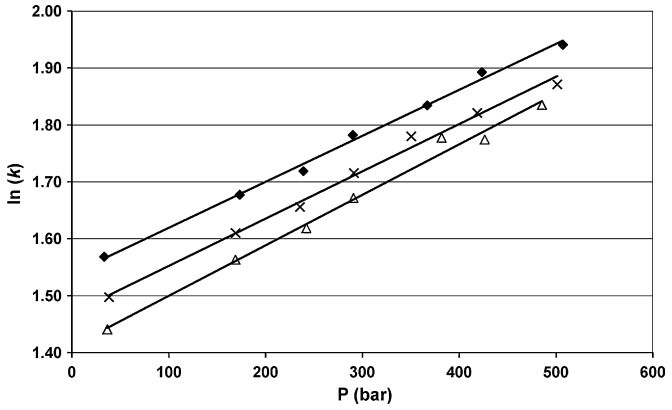

Fig. 3. Plot of ln k vs P for large polar compounds. Conditions: XBridge C_{18} BEH, mobile phase 25% acetonitrile–75% water at 0.2 mL/min, UV detection at 254 nm. Hydrocortisone (diamonds; $y = 0.000808x + 1.54$, $R^2 = 0.995$); prednisone (crosses; $y = 0.000820x + 1.47$, $R^2 = 0.995$) and prednisolone (triangles; $y = 0.000850x + 1.42$, $R^2 = 0.991$).

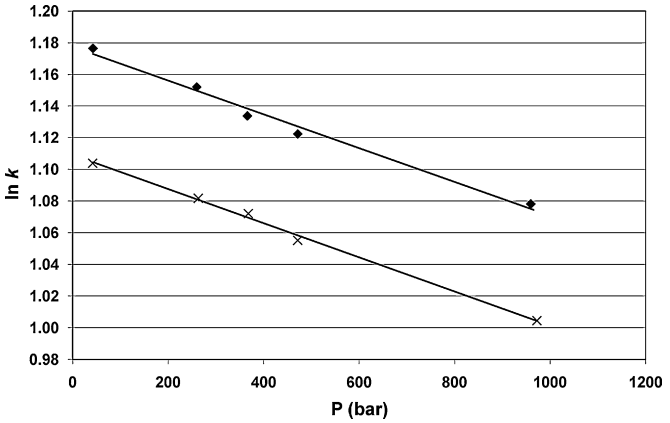

Fig. 4. Plot of ln k vs P at 0.6 mL/min for amitriptyline (diamonds; $y = -0.000106x + 1.18$, $R^2 = 0.982$) and nortriptyline (crosses; $y = -0.000108x + 1.11$, $R^2 = 0.992$). Conditions: Atlantis Silica, 5 μm, 50 mm × 2.1 mm column, 30 °C, 1 μL injection volume, mobile phase 90% acetonitrile–10% water with 5 mM ammonium formate at pH 3, flow rate at 0.6 mL/min, detection at 254 nm.

a flow rate of 0.2 mL/min. The linearity of the fits was good with correlation coefficients better than 0.99. The increases obtained at 0.2 mL/min were 50%, 51% and 53% for hydrocortisone, prednisone and prednisolone respectively, which have rather similar structures and MW. These results are comparable with the tricyclic bases amitriptyline (MW 277), nortriptyline (MW 263) and protriptyline (MW 263) which were protonated under the conditions of study and previously were shown to give increases of 50–53%, although these values were obtained with slightly different mobile phase compositions and flow rates to the corticosteroids [20]. Nevertheless, the result for amitriptyline in Table 1 with 25% ACN and flow 0.2 mL/min (53% increase in k for a 500 bar pressure increase) was obtained under the same conditions as for the corticosteroids. Thus it appears that neutral polar molecules can also exhibit considerable pressure-induced retention increases. However, the increased molecular size of the corticosteroids compared with the tricyclic bases is contributory to the retention increases observed. Our studies with weak bases (see below) have enabled the investigation of the same molecule in charged and neutral state, to investigate further the influence of ionisation on pressure-retention effects.

3.4. Influence of pressure on retention of bases in HILIC

HILIC has a completely different separation mechanism to that in RP-LC. Alpert [33] suggested that the mechanism mostly involves partitioning between the bulk mobile phase and a layer of mobile phase enriched with water and partially immobilised on the stationary phase. Adsorption and ion exchange with ionised silanols on silica-based columns are also likely to be contributory mechanisms. Therefore, it might be expected that the effect of pressure on retention would be different in HILIC and in RP-LC. Certainly, an increase in retention time with increasing pressure as a result of loss of part of the hydration layer and decrease in solvated molecular volume is not expected in HILIC, as the solute does not enter a hydrophobic environment on transferring from the mobile to the stationary phase.

Table 3 and Fig. 4 summarise the results obtained for two charged basic compounds, amitriptyline and nortriptyline using a bare silica column with an acetonitrile–ammonium formate buffer pH 3 (90:10, v/v). Recall that in RP chromatography, k increased by ~50% for these solutes for a pressure increase of 500 bar. In comparison, Table 3 shows *decreases* in retention of ~5% for either solute for a 500 bar pressure increase. It is indeed possible that these solutes are more hydrated in the "stationary phase", i.e. the water layer, than they are in the mobile phase, resulting in an increase in the solute molecular volume. According to Eq. (1), retention is therefore predicted to decrease with increasing pressure. This result can also be envisaged by considering the principle of Le Chatelier. The system will move to counteract the imposed increase in pressure, by decreasing its solvated molecular volume which can be accomplished by the solute residing more in the mobile phase as the pressure increases.

3.5. Influence of pressure on retention of weak bases when partially ionised

So far, all our studies have been focused on the effect of pressure using a mobile phase pH which resulted in solutes being either fully ionised, or fully neutral. In all cases in RP chromatography, we have observed an increase in retention when an increase in pressure is applied. In the light of the results reported by Tanaka and co-workers [21], we wished to understand to what extent pressure-induced changes in solute pK_a could influence retention, using the higher pressures available in our system. Weak bases such as pyridine and aniline derivatives were chosen for this study, as their

Table 3
Changes in k with pressure for two bases using HILIC.

Compound	F (mL/min)	Average column P with no restriction capillary (bar)	Average column P range (bar)	k with no restriction capillary	Slope of ln k vs P plot	Intercept	Correlation (R^2)	% change in k for a 500 bar P increase	ΔV (cm^3/mol) at 30 °C
Amitriptyline	0.4	28	28–641	3.25	−0.0000973	1.18	0.961	−4.7%	2.5
	0.5	35	35–809	3.27	−0.000104	1.18	0.972	−5.1%	2.6
	0.6	42	42–962	3.24	−0.000106	1.18	0.982	−5.2%	2.7
Nortriptyline	0.4	28	28–648	3.06	−0.000112	1.12	0.936	−5.4%	2.8
	0.5	35	35–816	3.06	−0.000111	1.11	0.969	−5.4%	2.8
	0.6	42	42–973	3.02	−0.000108	1.11	0.992	−5.3%	2.7

pK_a values lie in the middle of the pH range of operation of RP columns. Such compounds would also allow the study of the effects of pressure on retention of the same solute in completely ionised or non-ionised states, enabling observation of the effect of solute charge alone on retention changes with pressure.

Three pyridine derivatives were studied: 2-ethylpyridine, 3-ethylpyridine and 4-ethylpyridine, with pK_a of 5.89, 5.80 and 5.87 respectively. A mobile phase of ACN–water (10:90, v/v) containing 0.025 M KH_2PO_4 was chosen and adjusted to two different $_w{}^wpH$ values: pH 5.8, close to the solutes' pK_a, where a change of ionisation is expected with pressure, and pH 8, where the analytes are completely neutral. Unfortunately, it was impossible to study in addition the effect of pressure at low pH for these particular analytes when completely ionised, due to the very low retention obtained with these rather hydrophilic bases. For example, at pH 2.8 with the same mobile phase conditions, the retention factors of the three derivatives were around 0.1.

As expected, retention factors of the pyridine derivatives were greater at pH 8 than at pH 5.8 as the analytes are present entirely as the neutral species (see Table 4). In this condition of high pH, an increase in retention of approximately 10% was observed for a 500 bar pressure increase, similar to values observed previously with other small polar neutral solutes. The values of ΔV were all negative under these conditions. However, Table 4 shows that at pH 5.8, retention *decreased* for all 3 compounds as pressure was increased. For a pressure increase of 500 bar, retention factor decreases of 13%, 8% and 16% were obtained for 2-ethylpyridine, 3-ethylpyridine and 4-ethylpyridine respectively (see Table 4 at 0.2 mL/min).

The effects of pressure on ionisation constants have been discussed by a number of authors [34–36]. In every case, the ionisation of an electrically neutral acid or base in aqueous solution involves a contraction, and thus ionisation is enhanced by increase of pressure [34]. This effect can be rationalised by considering the principle of Le Chatelier, as the system moves to counteract the increase in pressure. The explanation of this contraction (electrostriction) is the strong interactions that exist between charged ions and the solvent (water). The electric fields of the ions strongly attract the dipolar solvent molecules and compress them locally to a higher density than they had around the parent molecules. Thus the pK_a of the ethylpyridines is increased as pressure increases. As pointed out by Tanaka [21], the effect of pressure on the buffer must also be considered. The ionisation of phosphate should also be increased, causing a decrease in the pH of the mobile phase as the pressure is raised. Both of these effects act in the same direction to increase the protonation of the ethylpyridines with pressure. As solutes are largely retained by hydrophobic retention processes, an increase in ionisation of the solute will result in a decrease in retention. The effect of increased solute protonation must act in opposition, and be greater than the effect of the change in molar volume of the solute as it transfers from the mobile to the stationary phase, as the latter effect causes retention to increase with pressure. These results are in agreement with those of Tanaka [21], who observed a decrease in retention for compounds such as 2,6-dimethylpyridine (pK_a 7.35) under similar mobile phase conditions with a C_{18} column. This example is interesting as 2,6-dimethylpyridine has the same molecular weight and similar polarity to the pyridine derivatives used in the present study. For example, Tanaka observed a decrease of 3.9% in retention of 2,6-dimethylpyridine for a pressure increase of about 100 bar in acetonitrile–phosphate buffer pH 6.8 (20:80, v/v); while we observed, for the same pressure increase, a decrease in retention of 2.8%, 1.6% and 3.4% for 2-ethylpyridine, 3-ethylpyridine and 4-ethylpyridine respectively in the conditions previously mentioned, i.e. acetonitrile–phosphate buffer pH 5.8 (10:90, v/v) at 0.2 mL/min. The experimental conditions in the study here are not exactly the same as the ones used by Tanaka (% in

Table 4
Changes in k with pressure for pyridine derivatives using ACN–phosphate buffers (10:90, v/v) at $_w{}^wpH$ 5.8 and pH 8.0.

pH mobile phase	Compound	F (mL/min)	Average column P with no restriction capillary (bar)	Average column P range (bar)	k with no restriction capillary	Slope of ln k vs P plot	Intercept	Correlation (R^2)	% change in k for a 500 bar P increase	ΔV (cm^3/mol) at 30 °C
5.8	2-Ethylpyridine	0.1	16	16–174	8.53	−0.000264	2.15	0.966	−12.4%	[a]
		0.2	32	32–357	8.55	−0.000282	2.16	0.999	−13.2%	[a]
		0.3	48	48–527	8.54	−0.000298	2.16	0.999	−13.8%	[a]
	3-Ethylpyridine	0.1	16	16–174	11.3	−0.000137	2.68	0.984	−6.6%	[a]
		0.2	32	32–357	11.3	−0.000162	2.69	0.996	−7.8%	[a]
		0.3	48	48–527	11.3	−0.000179	2.69	0.994	−8.6%	[a]
	4-Ethylpyridine	0.1	16	16–174	14.5	−0.000334	2.43	0.989	−15.4%	[a]
		0.2	32	32–357	14.6	−0.000347	2.44	0.999	−15.9%	[a]
		0.3	48	48–527	14.6	−0.000362	2.45	0.999	−16.6%	[a]
8.0	2-Ethylpyridine	0.1	16	16–171	14.1	0.000201	2.64	0.958	10.6%	−5.1
		0.2	36	36–361	14.1	0.000197	2.64	0.996	10.4%	−5.0
		0.3	50	50–523	14.2	0.000182	2.65	0.994	9.5%	−4.6
	3-Ethylpyridine	0.1	16	16–171	20.3	0.000195	3.01	0.961	10.2%	−4.9
		0.2	36	36–361	20.4	0.000201	3.01	0.993	10.6%	−5.1
		0.3	50	50–523	20.5	0.000186	3.01	0.993	9.7%	−4.7
	4-Ethylpyridine	0.1	16	16–175	20.7	0.000230	3.03	0.843	12.2%	−5.8
		0.2	34	34–359	20.9	0.000192	3.04	0.925	10.1%	−4.8
		0.3	49	49–529	21.0	0.000178	3.04	0.942	9.3%	−4.5

[a] Changes in k are due to both changes in solute ionisation and changes in molar volume.

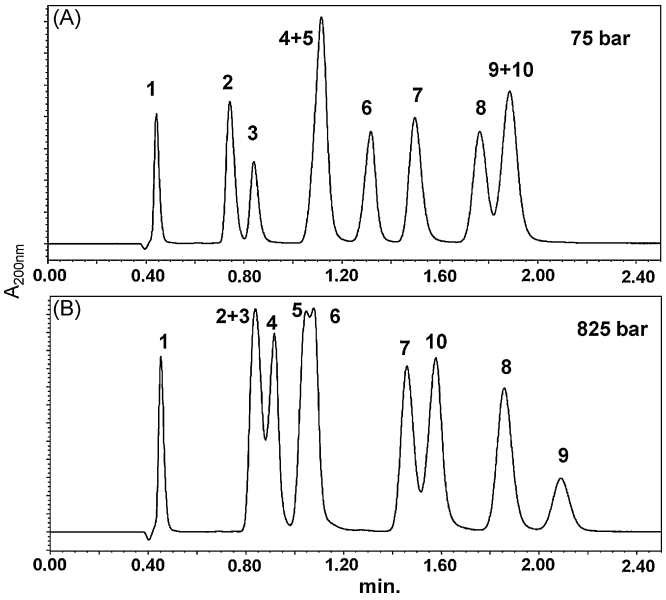

Fig. 5. Chromatograms of a mixture of ionisable compounds at different average column pressures: (a) 75 bar (b) 825 bar obtained without and with a 25 cm restriction capillary respectively. Conditions: XBridge C_{18} BEH, mobile phase acetonitrile–0.025 M potassium phosphate pH 5.8 (15:85, v/v), flow 0.3 mL/min, UV detection 200 nm. Peaks: 1, thiourea; 2, 2-methylbenzylamine; 3, pyridine; 4, 2,6-lutidine; 5, 2-picoline; 6, 2,4-lutidine; 7, 3-picoline; 8, aniline; 9, benzyl alcohol; 10, 3,4-lutidine.

acetonitrile for example) but the results are obviously very similar although the present instrumentation allows higher pressures and therefore greater changes in retention with pressure to be observed.

Considering that changes in k for ionised compounds depend on the mobile phase pH and on the analyte pK_a, it should be possible to obtain selectivity effects between these types of compounds, merely by increasing the pressure of the separation. Fig. 5 shows the separation of a mixture of various ionisable compounds pyridine (pK_a 5.27), 2,6-lutidine (pK_a 6.75), 2-picoline (pK_a 5.97), 2,4-lutidine (pK_a 6.74), 3-picoline (pK_a 5.52), aniline (pK_a 4.62; pK_a values from [37–40]), 2-methylbenzylamine pK_a (9.50), 3,4-lutidine (pK_a 6.5; pK_a from [25]) together with a neutral compound (benzyl alcohol) using acetonitrile-0.025 M phosphate buffer pH 5.8 (15:85, v/v) at 75 bar (no restriction capillary) and at 825 bar (with 25 cm restriction capillary). While the retention of analytes with a pK_a around the mobile phase pH (peaks 3, 4, 5, 6, 7 and 10) decreases when the pressure is increased; retention of benzyl alcohol (peak 9) and those analytes with a pK_a further from the mobile phase pH (peaks 2 and 8) increases with increasing pressure. These different changes in retention by increasing the pressure cause the separation of peaks which were co-eluting (4-5 and 9-10), the co-elution of peaks which were separated (2,3,4 and 5-6), and a reversal in the elution order of peaks 8 and 10 (aniline/3,4-lutidine). Pressure is thus potentially another parameter that could be used to adjust the selectivity of separations. Alternatively, separations may not be exactly the same on columns of different particle size due to their different operating pressures, even if the stationary phase chemistry is identical. In reversed-phase chromatography, an increase in pressure typically increases retention, while the accompanying thermal effects [1,18] decrease retention. Thus in practice when comparing the selectivity of for example, a 5 μm and a sub-2 μm particle column, the effects may partially cancel out.

3.6. Influence of pressure on retention of bases when ionised and neutral

Polar basic compounds are likely to be considerably hydrated. Charged species are more heavily hydrated than the uncharged species [41]. We performed a further study with 2-ethylaniline, 3-ethyl aniline and 4-ethylaniline (pK_a 4–5), as we wished to investigate the effect of pressure on retention of the same compounds when fully protonated and fully neutral, which had not been possible with the pyridine derivatives. Such an experiment should also throw light on whether ionisation of the compound gives rise to larger changes in retention with pressure, as would be expected if partial loss of the hydration layer was involved as the solute enters the stationary phase. It is important that the same % of water and organic modifier are used in the experiments at different pH values, as we have shown above that this is a factor affecting the change in retention of these solutes with pressure. It proved difficult to find suitable analytes that gave sufficient retention for the protonated base but not excessive retention for the neutral base with the same organic modifier/aqueous buffer concentrations. However, this condition was reasonably satisfied by these substituted anilines in conjunction with a mobile phase containing 10% acetonitrile. The solutes should be almost entirely protonated at pH 1.5 where they generated k values (in the absence of restriction capillaries) in the range 2.2–3.7, and almost entirely neutral at pH 7.2 where k values ranged from 32.8 to 41.4 (see Table 5). It would have been interesting to perform this experiment also at an intermediate pH between 4 and 5, i.e. around the analytes' pK_a; unfortunately, phosphate is not a suitable buffer for this range of pH (pK_{a1} 2.15 and pK_{a2} 7.20 [31]). We did not wish to substitute a different buffer which would have complicated the interpretation of the results. The plots of ln k vs P showed an acceptable linearity (R^2 between 0.89 and 0.99, Table 5). Although the absolute difference between the results at the two different pHs is not large, there is clearly less increase in retention with a 500 bar pressure increase when the analytes are fully neutral (8–9% for the three solutes) compared with when they are fully ionised (11–15%); the increase is 65%, 51% and 36% for 2-ethylaniline, 3-ethylaniline and 4-ethylaniline respectively. The results lend weight to the hypothesis that larger increases in retention with pressure are expected from ionised solutes than for polar neutral solutes, even though the increases for the latter can nevertheless be substantial, especially if the solutes have a high molecular weight. This result can be explained due to the likelihood of more extensive hydration of the

Table 5
Changes in k with pressure for aniline derivatives using ACN–phosphate buffers (10:90, v/v) at wwpH 1.5 and 7.2.

pH mobile phase	Compound	Average column P with no restriction capillary (bar)	Average column P range (bar)	k with no restriction capillary	Slope of ln k vs P plot	Intercept	Correlation (R^2)	% change in k for a 500 bar P increase	ΔV (cm^3/mol) at 30 °C
1.5	2-Ethylaniline	27	27–472	2.16	0.000281	0.782	0.891	15.1%	−7.1
	3-Ethylaniline	27	27–472	3.45	0.000234	1.24	0.915	12.4%	−5.9
	4-Ethylaniline	28	27–488	3.66	0.000210	1.31	0.891	11.1%	−5.3
7.2	2-Ethylaniline	48	48–400	32.7	0.000175	3.48	0.982	9.1%	−4.4
	3-Ethylaniline	47	47–402	39.6	0.000158	3.67	0.986	8.2%	−4.0
	4-Ethylaniline	47	47–401	41.4	0.000157	3.72	0.993	8.2%	−4.0

Table 6
Comparison of changes in k with pressure for bases with XBridge columns C1 and C18.

Compound	Mobile phase conditions	Column	Average column P with no restriction capillary (bar)	Average column P range (bar)	k with no restriction capillary	Slope of ln k vs P plot	Intercept	Correlation (R^2)	% change in k for a 500 bar P increase	ΔV (cm^3/mol) at 30 °C
Propranolol	30% ACN 70% water with 0.025 M phosphate buffer pH2.7 0.2 mL/min	C_1	21	21–386	1.18	0.000328	0.163	0.985	17.8%	−8.3
		C_{18}	28	28–355	2.17	0.000682	0.755	0.997	40.7%	−17.2
Diphenhydramine		C_1	21	21–386	1.90	0.000322	0.636	0.984	17.4%	−8.1
		C_{18}	28	28–355	3.33	0.000667	1.19	0.996	39.6%	−16.8
Protriptyline		C_1	21	21–386	3.26	0.000437	1.18	0.987	24.4%	−11.0
		C_{18}	28	28–355	6.72	0.000911	1.88	0.997	57.7%	−23.0
Nortriptyline		C_1	21	21–386	3.68	0.000430	1.30	0.986	24.0%	−10.8
		C_{18}	28	28–355	8.23	0.000926	2.08	0.997	58.9%	−23.3
Amitriptyline		C_1	21	21–386	4.01	0.000425	1.38	0.986	23.7%	−10.7
		C_{18}	28	28–355	9.30	0.000921	2.21	0.997	58.5%	−23.2
Anthracene	70% ACN 30% water 0.4 mL/min	C_1	29	29–547	0.974	0.0000564	0.0821	0.938	2.9%	−1.4
		C_{18}	30	30–468	3.65	0.000232	1.61	0.994	12.3%	−5.8
Pyrene		C_1	29	29–547	1.088	0.0000237	−0.0268	0.852	1.2%	−0.6
		C_{18}	30	30–468	5.05	0.000295	1.29	0.991	15.9%	−7.4

ionised solutes, leading to a greater loss in molar volume when the compound enters the hydrophobic environment of the stationary phase.

3.7. Influence of the carbon chain of the stationary phase on the pressure-induced changes in retention

If retention increases with increased pressure in RP-LC are caused by loss of solute solvation on entering the stationary phase, the effect might be expected to be different on bonded phases of different chain length. For example, the contact area with a C_{18} phase is larger than the contact area with a C_1, thus less solvent is released with the C_1, thus the pressure effect should be smaller.

Some ionised strong bases (propranolol, diphenhydramine, amitriptyline, nortriptyline and protriptyline) and some neutral compounds (anthracene and pyrene) were analysed in the same conditions of mobile phase as in our previous study [20], i.e. acetonitrile–0.025 M phosphate buffer pH 2.7 (30:70, v/v) for bases and acetonitrile–water (70:30, v/v) for the more hydrophobic neutral solutes. Table 6 summarises the changes in retention obtained for both C_1 and C_{18} columns. Changes in k with pressure clearly decrease by using a C_1 column instead of a C_{18} column. For example, retention of amitriptyline increased by 59% for a 500 bar pressure using a C_{18} column, compared with only 24% on a C_1 column. This behaviour was also observed for the neutral compounds, whose retention increased by 1–3% with the C_1 column instead of 12–16% with C_{18} column. However, even if changes in k are much lower with the C_1 column, they are still quite considerable and certainly not negligible for ionised compounds. These results are broadly in agreement with the mechanisms that we have proposed.

4. Conclusion

We have confirmed that the large increases in retention with pressure for polar and ionised solutes were not due to some unusual property of the hybrid inorganic–organic RP column used previously. Very similar results were obtained also for a conventional silica C_{18} phase. All the results in the present study support the hypothesis that these increases in retention are due to a reduction in the solute molar volume as it transfers from the mobile to the stationary phase, and that this could be caused by loss of the solute hydration layer when it enters the hydrophobic network of the bonded phase chains.

The pressure-induced increases in retention for these solutes in RP-LC appear to increase as the mobile phase water content increases from 50 to 75% water, followed by a decrease at higher water concentration. If the increases in retention with pressure are due to partial loss of the solute hydration layer on entering the stationary phase, the initial increase could be explained by greater hydration of these solutes as the mobile phase water content increases. It is possible that the subsequent decrease in retention at high water content may be due to changes in the structure of water–ACN mixtures, or to the increased viscosity of mobile phases of high water content giving small frictional heating effects. Polar neutral compounds can also show large increases in retention with pressure, especially for large molecules. Experiments using weak bases in the ionised and non-ionised states however suggest that somewhat larger increases are obtained for ionised solutes of the same molecular weight.

Retention increases with pressure were shown to be considerably smaller for a C_1 phase compared with a C_{18} phase because the contact area between solute and stationary phase is smaller with C_1 chains. Using a bare silica HILIC column, *decreases* in retention with pressure for ionised bases were noted. This result is consistent with the idea that the solute may become more hydrated in the water

layer associated with the stationary phase in HILIC, compared with the solvation in the mobile phase.

Finally, we have confirmed that retention changes with pressure in the region of solute pK_a can be caused by pressure-induced pK_a changes of the solute and mobile phase buffer. However, this effect appears to be an entirely separate phenomenon compared with those that we have observed for fully ionised or for neutral solutes and requires further study.

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