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Effect of counter-anion concentration on retention in high-performance liquid chromatography of protonated basic analytes

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Abstract

The influence of acid and salt concentration in the mobile phase on the retention of basic analytes has been studied. An increase in the retention of fully protonated analytes with increasing the concentration of inorganic additives was found. The addition of salt, such as perchlorate, trifluoroacetate, and phosphate, leads to the increase of retention for fully protonated analytes while mobile phase pH remains constant. The observed effect was attributed to the interaction of protonated analytes with the counter-anion of acid or salt, which leads to the disruption of the analyte solvation shell and the increase of its hydrophobicity and corresponding increase of retention. A mathematical model for the description of the influence of counter-anion concentration on analyte retention is proposed.

Keywords: Mobile phase composition; Mathematical modeling; Picoline; Lutidines; Pyridines; Anilines; Benzylamines

1. Introduction

Development and optimization of reversed-phase high-performance liquid chromatography (HPLC) separation methods for a mixture of ionizable organic compounds (primarily pharmaceuticals) is a challenge. Retention of these ionizable components is dependent on many parameters, such as eluent type and composition [1,2], type of stationary phase [3], presence of accessible residual silanols [4,5], eluent pH [6–10], buffer type and concentration [11,12]. Also, secondary equilibria in the HPLC system were shown to have a significant effect on analyte retention [13].

In the previous paper [14] we discussed the effects of buffer type and concentration on the retention of substituted amines and pyridines in a low pH region, where complete ionization of these compounds is achieved. Observed effects of systematic increase in retention with the increase of counter-anion concentration in a low pH region was attributed to the influence of acidic modifier counter-anions on the degree of solvation of basic analytes. Similar effects were obtained for a basic pharmaceutical compound with mobile phase pH change from 3 to 1 using perchloric, trifluoroacetic, nitric and phosphoric acids on a silica-based crown ether column [15]. The authors attributed this effect to the type and increase of the acidic modifier counter-anion concentration, which affected the analyte solvation. The retention of propranolol (basic racemate) on a Chiralcel OD-R column [16] was shown to increase to different degrees when various counter-anions, such as perchlorate, and nitrate were used as mobile phase additives. Other primary, secondary and tertiary amines showed similar retention effects [16]. An

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increase in retention of basic ophthalmic compounds on a C_{18} column was obtained with an increase of phosphate, trifluoroacetate, and perchlorate counter-anion concentrations [17]. All of these effects are attributed to the ionic interaction of protonated analyte with oppositely charged species, which result in the disruption of the analyte solvation.

It has been shown that basic analytes are solvated and that the hydration stabilizes the protonated form of amines, with more solvating water molecules resulting in a larger stabilization [18]. Basic compounds that have an increasing number of substituents or substituents of increasing size near the amino group are solvated to different degrees in solution, which may be due to limitations in hydrating their polar sites. Molecular mechanics [19,20], STO-3G ab initio [21] and electrostatic potential calculations [22] have been applied for the investigation of the hydration effects on the basicity of amines, and it has been shown that the pK_a of amines is influenced by conformational changes in solution as well as by potential contributions of water molecules bound strongly to the base. It has also been shown that depending on the nature of the analyte, methanol or acetonitrile could also effect the solvation of the analyte [23].

Since basic analytes in solution are solvated by water as well as organic eluent components, the composition of the immediate surroundings of a solute may differ from the composition of the bulk mixture and this may be explained in terms of preferential solvation. Preferential solvation is attributable to the presence of a molecular excess of either of the eluent components in these surroundings [24]. The variation of the preferential solvation is mostly related to the structural features of these mixtures [25]. However, if the solute shows no preference for the solvent molecules, the solvent composition in the immediate neighborhood of the solute is the same as in the bulk liquid. It has been shown that there is preferential solvation of hydrogen ion, acetate ions [28], fluoroquinolones [29], other buffer species [30] including tartrate, citrate, phthalate and phosphate ions in acetonitrile–water mixtures [26,27]. Migron and Marcus have described the overall picture of the preferential solvation of water–acetonitrile mixtures from various studies [31].

The influence of acidic modifier counter-anion concentration on the retention of ionized basic analytes is attributed to the solvation equilibria of the ionic species. The protonated (ionic) basic analyte is solvated with water molecules, and thus relatively more hydrophilic. An increase of acidic modifier counter-anion concentration in the mobile phase disrupts the analyte solvation shell due to ion association. Disruption of the analyte solvation results in an increase in the analyte hydrophobicity and its retention. The desolvation of a protonated basic analyte was determined to be a function of the counter-anion concentration and is denoted as the chaotropic effect. Theoretical description of the effect of ion association on the analyte retention is suggested.

A model is developed to describe the effect of the analyte desolvation process upon addition of counter-anions to the mobile phase. The influence of the type and concentration of organic eluent in the mobile phase on these effects will also be investigated.

2. Experimental

2.1. Apparatus

The chromatographic system used was a Model 1100 HPLC from Hewlett-Packard (HP) (Little Falls, DE, USA). The chromatograms were processed using HP software. The column used was a Zorbax Eclipse XDB-C_{18} (Hewlett-Packard), 150× 4.6 mm I.D., particle diameter 5 μm, bonding density 3.4 μmol/m^2. The Eclipse XDB-C_{18} column has a nominal surface area of 180 m^2/g, and a pore size of 80 Å.

The column temperature was controlled by a circulating water-bath Brinkman Model RC6 Lauda (Lauda-Konigshofen, Germany). The pH was measured using a Fisher Accumet pH meter 15 on the aqueous eluent component before the addition of the organic modifier. The electrode was calibrated with pH 1.0, 2.0, 4.0, 7.0 and 10.0 standard solutions.

2.2. Chemicals

Perchloric acid, acetonitrile (HPLC grade) and methanol (HPLC grade) were obtained from Sigma (Milwaukee, WI, USA). Sodium perchlorate was

purchased from Fisher Scientific (Fairlawn, NJ, USA). All aqueous mobile phases were filtered using a Whatman nylon 66 membrane filter (Fisherlane). The following basic compounds were used: aniline (Baker), *N*-methylaniline (Eastman, TN, USA), 2-ethylpyridine, 3-ethylpyridine, 4-ethylpyridine, 2,4-dimethylpyridine, 2,6-dimethylpyridine, 3,4-dimethylpyridine, 3,5-dimethylpyridine (Aldrich), benzylamine and *R*-methylbenzylamine (Lancaster Labs., Lancaster, PA, USA).

2.3. Chromatographic conditions

The retention data were recorded at controlled temperatures between 25 and 45°C using isocratic conditions with a flow-rate of 1 ml/min using the Zorbax Eclipse XDB-C_{18} column. UV detection was at 254 nm for the entire study. Acetonitrile and methanol were used as the organic modifiers. The eluent composition was varied from 90:10 (aqueous–organic) to 50:50 (aqueous–organic).

All analyte solutions with the exception of the benzylamines were prepared by their dissolution in the eluent to give a concentration of 0.1–0.2 mg/ml. Benzylamines were dissolved in water–acetonitrile (70:30) to give a concentration of 0.2 mg/ml. Injections of 1–5 μl of these solutions were made.

The t_0 values obtained for the Zorbax XDB-C_{18} column were determined using the minor disturbance method within the temperature range of 25–45°C. The retention factors calculated were the averages of triplicate injections showing relative standard deviations (RSDs) less than 1.3%. Also, a test mixture of aniline and phenol was used as system suitability check before and after each experiment to monitor the performance and the stability of the column.

3. Results and discussion

All the analytes studied show an increase of their retention with increase of the counter-anion concentration in the mobile phase at low pH (pH 2.6–pH 2.9) where analytes were fully protonated. The experimental dependencies of the analyte retention on the counter-anion concentration are shown in Figs. 1 and 2 (points). Major effects on the analyte retention are observed at low counter-anion concentration. A slight increase of the amount of counter-anions in a low concentration region results in significant increase of the analyte retention. However, in a high concentration region even significant increase of counter-anion content shows almost no effect on the analyte retention.

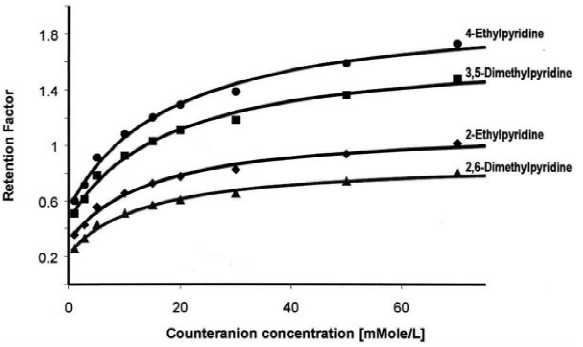

Fig. 1. Experimental retention factors for mono- and disubstituted pyridine compounds (points) versus perchlorate concentration and corresponding curves obtained using Eq. (10) in water–acetonitrile (90:10) eluent. Conditions: concentration region 1–70 m*M* of perchlorate anion in water. Column: 15×0.46 cm Zorbax XDB-C_{18}; mobile phase: acetonitrile–water (90:10) adjusted with perchloric acid and $NaClO_4$ and/or solely perchloric acid, pH 2.6–2.9; flow-rate, 1.0 ml/min; temperature: 25°C, UV detection at 254 nm; sample: 1 μl injection.

As was shown in the previous paper [14], this

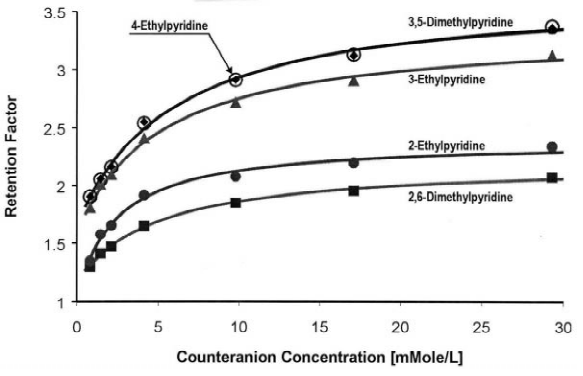

Fig. 2. Experimental retention factors for mono- and disubstituted pyridine compounds (points) versus perchlorate concentration and corresponding curves obtained using Eq. (10) in water–methanol (90:10) eluent. Concentration region 0.8 m*M*–29 m*M* of perchlorate anion in water. Column: 15×0.46 cm Zorbax Eclipse XDB-C_{18}; mobile phase: methanol–water (90:10) adjusted with perchloric acid, pH 1.5–2.9; flow-rate, 1.0 ml/min; temperature: 25°C, UV detection at 254 nm; sample: 1 μl injection.

effect is related to the influence of the counter-anion of the acidic modifier on the analyte solvation, and is independent on the mobile phase pH, as far as complete protonation of the basic analyte is achieved. Analyte interaction with counter-anion of acidic modifier causes a disruption of the analyte solvation shell, thus affecting its hydrophobicity. Less solvated organic basic analytes are relatively more hydrophobic than corresponding analytes with nondisrupted solvation shells. Increase of the analyte hydrophobicity results in corresponding increase of retention. This process shows a ''saturation'' limit, when counter-anion concentration is high enough to effectively disrupt the solvation of all analyte molecules. A further increase of counter-anion concentration does not produce any noticeable effect on the analyte retention.

If the counter-anion concentration is low, some analyte molecules have a disrupted solvation shell, and some do not due to the limited amount of counter-anions present at any instant. If we assume an existence of the equilibrium between solvated and desolvated analyte molecules and counter-anions, this mechanism could be described mathematically.

The assumptions for this model are:

(1) Analyte concentration in the system is low enough that analyte–analyte interactions could be considered nonexistent.

(2) The chromatographic system is in thermodynamic equilibrium.

The analyte solvation–desolvation equilibrium inside the column could be written in the following form:

$$B_S^+ + A^- \Leftrightarrow B^+ \cdots A^- \quad (1)$$

Where B_S^+ is a solvated basic analyte, A^- is a counter-anion, $B^+ \cdots A^-$ is the desolvated ion-associated complex. The total amount of analyte injected is [B], analyte in its solvated form is $[B_s^+]$, and analyte in its desolvated form is denoted as $[B^+ \cdots A^-]$, indicating its interaction with counter-anions.

The expression for the equilibrium constant (K) of reaction (1) is:

$$K = \frac{[B^+ \cdots A^-]}{[B_S^+][A^-]} \quad (2)$$

The total analyte amount is equal to the sum of the amount of solvated and amount of desolvated analyte:

$$[B] = [B_S^+] + [B^+ \cdots A^-] \quad (3)$$

It is convenient to normalize the above equation and express the amount of analyte in solvated and desolvated form as a fraction of the total amount. The fraction of solvated analyte could be expressed as:

$$\theta = \frac{[B_S^+]}{[B]} \quad (4)$$

The fraction of the protonated analyte desolvated due to the interaction with counter-anions in the mobile phase could be expressed as:

$$1 - \theta = \frac{[B^+ \cdots A^-]}{[B]} \quad (5)$$

Substituting Eqs. (4) and (5) into Eq. (2) we can write an expression for the equilibrium constant:

$$K = \frac{1 - \theta}{\theta[A^-]} \quad (6)$$

solving Eq. (6) for θ (solvated fraction) we get:

$$\theta = \frac{1}{K[A^-] + 1} \quad (7)$$

Eq. (7) shows that the fraction of the analyte which remains solvated is dependent on the counter-anion concentration and desolvation equilibrium parameter. Corresponding expressions could be written for desolvated analyte, remembering that its fraction is expressed as $1-\theta$.

Completely solvated analyte has some retention factor (even if it is equal to 0), which we denote as k_s, while the corresponding retention factor for desolvated form we denote as k_{us}.

The overall retention factor of injected analyte is a sum of the retention factor of solvated form multiplied by the solvated fraction (θ) and the retention factor of the unsolvated form multiplied by the unsolvated fraction ($1-\theta$), or:

$$k = k_s\theta + k_{us}(1 - \theta) \quad (8)$$

Substituting θ in Eq. (8) from Eq. (7) we get:

Table 1
Retention factor and desolvation parameters obtained using perchlorate as the counter-anion and MeCN–water (10:90) eluent on Zorbax Eclipse XDB-C_{18} column at 25°C[a]

	2-Picoline	4-Picoline	2-Ethylpyridine	4-Ethylpyridine	2,6-Lutidine	3,5-Lutidine	Benzylamine	*R*-Methylbenzylamine
k_s	0.126	0.183	0.313	0.546	0.221	0.462	0.964	1.883
k_{us}	0.505	0.696	1.13	1.987	0.886	1.69	3.362	6.978
K	0.093	0.079	0.068	0.055	0.072	0.057	0.058	0.053

[a] Same conditions as in Fig. 1.

$$k = k_s\left(\frac{1}{K[A^-]+1}\right) - k_{us}\left(\frac{1}{K[A^-]+1}\right) + k_{us} \quad (9)$$

and the final form can be rewritten as:

$$k = \frac{k_s - k_{us}}{K[A^-]+1} + k_{us} \quad (10)$$

This is the final mathematical expression for the analyte retention dependence on the counter-anion concentration derived on the basis of the proposed model. This equation has three parameters: k_s is a ''limiting'' retention factor for solvated analyte, k_{us} is a ''limiting retention factor'' for desolvated analyte, and K is a desolvation parameter.

At a counter-anion concentration equal to 0, the analyte retention will be equal to k_s. Increase of the counter-anion concentration leads to the asymptotic approach of the analyte retention to k_{us}. Desolvation parameter K defines the slope of retention dependence in a low counter-anion concentration region.

Figs. 1 and 2 show the superposition of experimental retention factors for different basic analytes (points) and theoretical curves calculated using Eq. (10) and nonlinear curve-fitting. Parameters for Eq. (10) for each system are shown in Tables 1 and 2.

All experimental data fit to the proposed mathematical form of concentration dependence fairly well, as the RSD does not exceed 3%. This confirms the applicability of the proposed model for the description of observed chaotropic effects of acidic modifier counter-anion concentration on the retention of protonated basic analytes.

Eq. (10) is the general form that describes the effect of analyte desolvation on chromatographic retention. The specific solvation of the analyte and the counter-anion may be dependent upon the temperature and type of organic modifier and the modifier concentration. This may lead to different desolvation parameters for the same analyte in two different systems. For an analyte that has a greater desolvation parameter, the analyte is desolvated at lower concentrations of counter-anion as shown in Fig. 3.

3.1. Effect of type of eluent on the desolvation parameter

The retention dependencies of basic analytes on the concentration of perchlorate anion in the acetonitrile–water (10:90) and methanol–water (10:90) eluents were determined on a Zorbax XDB-C_{18} column. The graphs of retention factors for these dependencies are shown in Fig. 1 for the acetonitrile–water eluent and in Fig. 2 for methanol–water eluent. The best fits of the theoretical curves to the experimental data were obtained using Eq. (10) with a nonlinear regression program, MathCad 8. Good correlation of theoretical curves with experimental points suggests the applicability of solvation–de-

Table 2
Retention factor and desolvation parameters obtained using perchlorate as the counter-anion and methanol–water (10:90) eluent on Zorbax Eclipse XDB-C_{18} column at 25°C[a]

	2,4-Lutidine	3,4-Lutidine	2,6-Lutidine	3,5-Lutidine	2-Ethylpyridine	3-Ethylpyridine	4-Ethylpyridine	Aniline	*N*-Methylaniline
k_s	1.456	1.621	1.218	1.761	1.195	1.707	1.759	1.747	2.547
k_{us}	3.237	3.812	2.327	4.005	2.545	3.632	3.961	3.446	5.492
K	0.13	0.126	0.136	0.106	0.233	0.114	0.111	0.05	0.089

[a] Same conditions as in Fig. 2.

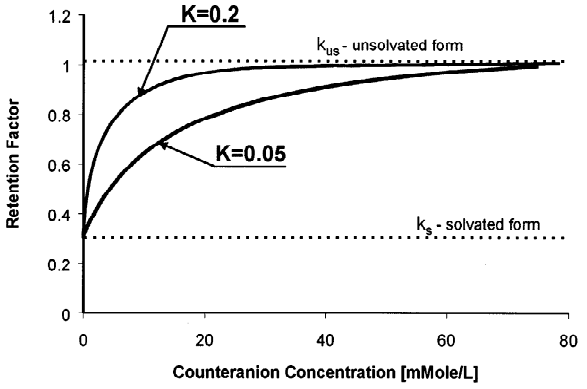

Fig. 3. Theoretical dependencies of retention factor on counter-anion concentration (m*M*). The higher the *K* value the greater the curvature of this dependence.

solvation equilibrium for the description of the counter-anion concentration effect on the analyte HPLC retention.

This procedure allowed the estimation of the desolvation parameter, *K*, and limiting retention factors, k_s, k_{us}, of the solute in the HPLC mobile phase. Parameters k_s, k_{us} and *K*, calculated using Eq. (10) for all analytes for two different organic modifiers used, are shown in Table 1 (acetonitrile) and in Table 2 (methanol).

Different limiting analyte retention factors, k_s and k_{us}, and desolvation parameters, *K*, were obtained in the two-eluent systems employed: methanol–water and acetonitrile–water. This may be attributed to specific analyte–solvent interactions. Methanol due to its ability to form hydrogen bonds may actually participate in the analyte solvation as opposed to acetonitrile. This was supported in the literature [31], where large positive values of the preferential solvation in a methanol–water binary system were obtained indicating that strong mutual interactions of the components are preferred over the self-interactions between methanol molecules. Therefore, analyte solvation with methanol would increase the analyte hydrophobicity and aid in the analyte retention process. In this system the presence of methanol molecules and increase of perchlorate anion concentration produced a synergistic effect on the analyte retention. Combinations of the same counter-anion with different solvents may not only lead to differences in the analyte solvation but to differences in the selectivity between components as shown in Figs. 1 and 2. The selectivity between 4-ethylpyridine and 3,5-dimethylpyridine is enhanced in the ''perchlorate–acetonitrile'' system when compared to the ''perchlorate–methanol'' system.

3.2. Effect of concentration of organic solvent on the solvation parameter

The dependencies of basic analyte retention on the concentration of perchlorate counter-anion were measured on Zorbax XDB-C_{18} column for different acetonitrile concentrations in the eluent. The pH of the aqueous portion of the mobile phase was adjusted to the same value for all experiments with perchloric acid and the concentration of perchlorate anion was varied by addition of $NaClO_4$. Fig. 4 shows the retention dependencies of a representative set of basic compounds at increasing concentration of perchlorate counter-anion analyzed at acetonitrile–water (90:10) at 35°C. Graphs of the retention dependencies of 2-ethylpyridine on the counter-anion concentration for all acetonitrile–water compositions used (10–50% of acetonitrile) are shown in Fig. 5. Similar dependencies were obtained for all other basic analytes studied.

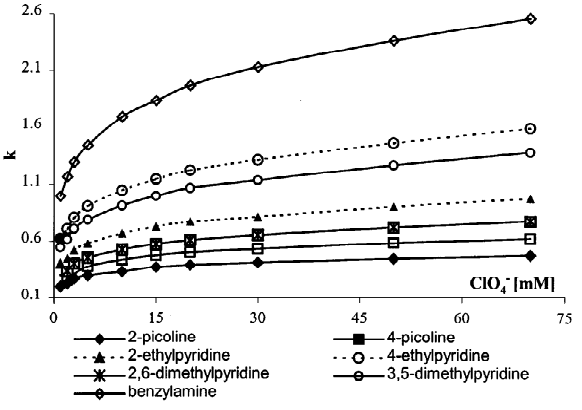

Fig. 4. Retention factor versus counter-anion concentration for pyridinal and benzylamine compounds. Conditions: concentration region 1–70 m*M* of perchlorate anion. Column: 15×0.46 cm Zorbax XDB-C_{18}; mobile phase: acetonitrile–water (90:10) adjusted with perchloric acid and $NaClO_4$ and/or solely perchloric acid, pH 2.6–2.9; flow-rate, 1.0 ml/min; temperature: 35°C, UV detection at 254 nm; sample: 1 μl injection.

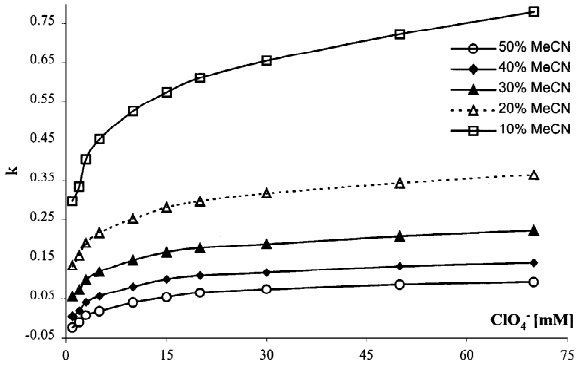

Fig. 5. Retention factor of 2,6-dimethylpyridine versus counter-anion concentration at different mobile phase compositions, 35°C. Conditions: concentration region 1–70 m*M* of perchlorate anion. Column: 15×0.46 cm Zorbax XDB-C_{18}; mobile phase: acetonitrile–water adjusted with perchloric acid and $NaClO_4$ and/or solely perchloric acid, pH 2.6–2.9. Water content varied from 50 to 90%; flow-rate, 1.0 ml/min; temperature: 35°C; UV detection at 254 nm; sample: 1 μl injection.

The desolvation parameter, *K*, for all measured dependencies is shown in Table 3. The increase of the acetonitrile content in the mobile phase causes an increase of the analyte desolvation parameter (Table 3) indicating that the same degree of desolvation is achieved at much lower concentrations of the perchlorate anion in the eluent with higher organic content. This may be attributed to the greater disruption of the analyte solvation by the acetonitrile molecules at increasing concentrations. Hence the increase of acetonitrile content is actually aiding in the desolvation process. At an acetonitrile mole fraction greater than 0.15 it could no longer be accommodated within the cavities of the structure of ordinary water [32–34]. Plots of the desolvation parameter versus acetonitrile concentration obtained at 35°C and 45°C are shown in Fig. 6a and b, respectively. The desolvation parameter increases predominately in the region of 10–40% acetonitrile. The observed effects show that the increase in acetonitrile content causes a greater disruption in the analyte solvation, and ultimately allows the limiting retention factor for the unsolvated form to be obtained at lower concentrations of counter-anion.

Another rationale is that with the increase of acetonitrile concentration in the eluent the dielectric parameter decreases. This permits the two oppositely charged species to be attracted to each other with a greater force, since force is inversely proportional to the dielectric parameter. Upon doing so, this permits

Table 3
The effect of acetonitrile concentration on the desolvation parameters of basic analytes[a]

Basic analyte	Acetonitrile concentration (%, v/v)				
	10	20	30	40	50
2-Picoline	0.089	0.149	0.174	0.21	0.168
4-Picoline	0.100	0.121	0.155	0.166	0.143
2-Ethylpyridine	0.073	0.117	0.153	0.166	0.152
4-Ethylpyridine	0.059	0.098	0.127	0.151	0.156
2,6-Dimethylpyridine	0.078	0.108	0.129	0.132	0.124
3,5-Dimethylpyridine	0.06	0.106	0.137	0.156	0.157
Benzylamine	0.057	0.074	0.107	0.12	0.103
R-Methylbenzylamine		0.064	0.089	0.104	0.119

[a] Same conditions as in Fig. 5.

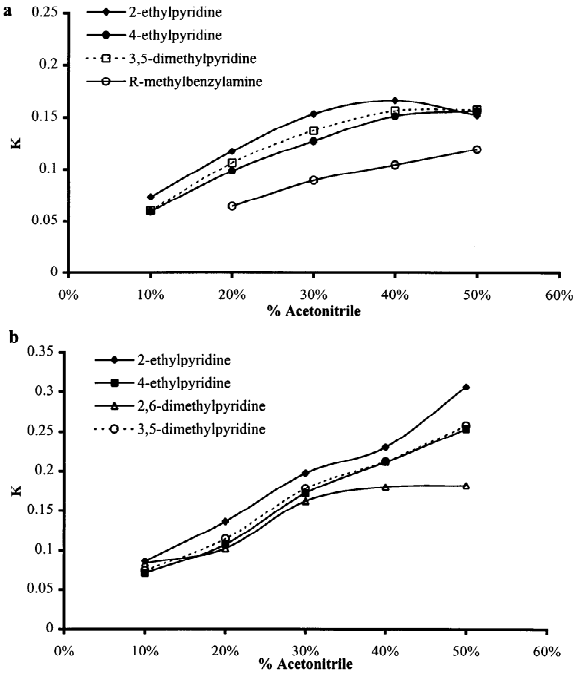

Fig. 6. (a) Dependence of desolvation parameter, *K*, versus the concentration of acetonitrile in the mobile phase at 35°C. (b) Dependence of desolvation parameter, *K*, versus the concentration of acetonitrile in the mobile phase at 45°C. Conditions: concentration region 1–70 m*M* of perchlorate anion. Column: 15× 0.46 cm Zorbax XDB-C_{18}; mobile phase: acetonitrile–water adjusted with perchloric acid and $NaClO_4$ and/or solely perchloric acid, pH 2.6–2.9. Water content varied from 50 to 90%; flow-rate: 1.0 ml/min, UV detection at 254 nm; sample: 1 μl injection.

greater analyte desolvation since as perchlorate anion approaches the protonated basic analyte this forces the expulsion of water from the analyte solvation shell.

4. Conclusion

The effect of counter-anion concentration on HPLC retention of protonated basic analytes is associated with ionic interactions of anions (from salt or acid) with positively charged analyte molecules. These ionic interactions lead to the disruption of the analyte solvation shell, increase of analyte hydrophobicity, and a corresponding increase in retention.

Mathematical interpretation of the effect of counter-anions on the analyte solvation–desolvation equilibrium allows the description of the analyte retention as a function of counter-anion concentration. Experimental verification of the proposed model confirms its applicability for all analytes studied.

The effect of different organic modifiers at various eluent compositions was also studied. The dependencies of calculated analyte solvation parameters on the concentration of organic modifier of the eluent appear to correlate with known studies of the preferential solvation process.

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