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Retention of Ionizable Compounds on HPLC. 8. Influence of Mobile-Phase pH Change on the Chromatographic Retention of Acids and Bases during Gradient Elution

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The relationships between retention and mobile-phase pH in gradient elution are studied for acids and bases. The apparent pH shift caused by the increasing amount of acetonitrile and methanol has been determined starting from a wide range of pH values. It is shown that good relationships between the retention of ionizable compounds and the pH of the aqueous buffer can be established if the same type of buffer (ammonium acetate in this work) is used for all pH points. Equations are proposed to fit the gradient retention data to the pH of the aqueous buffer. The proposed equation gives an account of the relative variation of the pK_a of the compound in the reference to the variation of the pH of the buffer as both parameters change during gradient elution.

The effect of gradient elution on the chromatographic retention of nonionizable compounds was studied by Schoenmakers.[1,2] The variation of the mobile-phase composition during gradient elution implies changes in the retention parameters of the eluted compound. The observed retention is a composite of the variation of the retention factor (k) of the compound with the mobile-phase composition and the variation of the mobile-phase composition with time (gradient).

Nowadays, fast generic gradient methods are frequently used in drug discovery quality control processes combined with mass spectrometric detection. The fast gradient generic methods are based on using short column, higher flow rates without too much loss of resolution.[3] These quality control methods usually apply a low-pH aqueous starting mobile-phase pH. Recently, the fast gradient method has been applied for assessment of lipophilicity of the molecules via deriving the chromatographic hydrophobicity index (CHI) from the gradient retention time.[4−7] Because many drug molecules show acid/base properties, the CHI lipophilicity is usually measured with three different starting mobile-phase pH values (pH 2, 7.4, and 10.5). However, the pure forms of the different acid/base species of some drugs cannot be achieved at any of these three pH values. Therefore, there is a need for fitting models to calculate the CHI lipophilicity of the different drug species (neutral and ionic) from CHI data at different starting pH values.

When the compound eluted has acid/base properties, the variation of the mobile-phase composition during gradient elution produces changes in the degree of ionization of the compound which contribute significantly to variation of retention. The change on the ionization of the compound depends on two parameters that change during compound elution: the pK_a value of the compound and the pH of the mobile phase. The models for gradient methods presently available do not consider the change of these parameters.

In this work, the influence of the change in mobile-phase pH and compound pK_a in the retention of several test compounds is studied. Equations are proposed to account for these changes during gradient elution and relate the retention of the compounds to the initial pH of the mobile phase. They shall be used to obtain CHI descriptors of the pure acid/base forms of drugs. The model can be implemented in optimization strategies for separations including acid/base compounds by gradient elution chromatography. A recent publication[8] remarks the usefulness of computer modeling and simulation to improve chromatographic methods development.

pH Measurement and pH and p*K* Scales in HPLC Mobile Phases. The different procedures for the measurement of pH in HPLC mobile phases and the different pH and pK scales to which they lead have been recently evaluated and compared for methanol/water[9] and acetonitrile/water.[10]

In isocratic elution, the most correct procedure consists of measuring the pH of the mobile phase after mixing the aqueous buffer and the organic modifier. The electrode system for pH

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(1) Schoenmakers, P. J.; Billiet, H. A. H.; Tijssen, R.; De Galan, L. *J. Chromatogr.* **1978**, *149*, 519−537.
(2) Schoenmakers, P. J.; Billiet, H. A. H.; De Galan, L. *J. Chromatogr.* **1979**, *185*, 179−195.
(3) Mutton, I. M. *Chromatographia* **1998**, *47*, 291−298.
(4) Valkó, K.; Bevan, C.; Reynolds, D. *Anal. Chem.* **1997**, *69*, 2022−2059.
(5) Du, C. My; Valkó, K.; Bevan, C.; Reynolds, D.; Abraham, M. H. *Anal. Chem.* **1998**, *70*, 4228−4234.
(6) Valkó, K.; Plass, M.; Bevan, C.; Reynolds, D.; Abraham, M. H. *J. Chromatogr., A* **1998**, *797*, 41−55.
(7) Valkó, K.; Du, C. My; Bevan, C.; Reynolds, D. P.; Abraham, M. H. *Curr. Med. Chem.* **2001**, *8*, 1137−1146.
(8) Molnar, I. *LC-GC Eur.* **2001**, *14*, 231−242.
(9) Canals, I.; Portal, J. A.; Bosch, E.; Rosés, M. *Anal. Chem.* **2000**, *72*, 1802−1809.
(10) Espinosa, S.; Bosch, E.; Rosés, M. *Anal. Chem.* **2000**, *72*, 5139−5200.

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measurement can be calibrated with the usual aqueous standards, which leads to the absolute pH scale ($^{s}_{w}$pH), or with standards in the same mixed organic solvent used as mobile phase, which in turn leads to the relative pH scale ($^{s}_{s}$pH). Both pH scales differ in the δ term, which is constant for each solvent (mobile-phase organic modifier and percentage) and has been determined for methanol/water and acetonitrile/water mobile phases.[9,10]

$$\delta = {}^{s}_{w}\mathrm{pH} - {}^{s}_{s}\mathrm{pH} \quad (1)$$

The parameters obtained in the fit of the retention of acids and bases to mobile-phase pH measured in any of these two rigorous pH scales show a direct relationship with the thermodynamic acid/base constants of the compound in the same scales ($^{s}_{s}$pK or $^{s}_{w}$pK).

Nevertheless, in gradient elution, it is not possible to fit the retention of acids and bases to the $^{s}_{w}$pH or $^{s}_{s}$pH of the mobile phase because these pH values change during the elution of the compounds. In this instance, the unique practical possibility is to fit retention to the pH value of a particular buffered mobile-phase composition. The most practical is to measure the pH value of the aqueous buffer before mixing it with the organic modifier, which leads to the aqueous pH scale ($^{w}_{w}$pH). The fits obtained with the $^{w}_{w}$pH scale are worse than those obtained with the $^{s}_{w}$pH or $^{s}_{s}$pH scales, unless buffers of the same type are used in the pH range where compound retention changes.[9,10]

The pH variation during gradient elution is different for each type of buffer, and thus, we have always used the same aqueous buffer of ammonium acetate in all experiments, to which formic acid or ammonia has been added to obtain the appropriate $^{w}_{w}$pH values. The acid/base pair acetic/acetate keeps the $^{w}_{w}$pH buffered between 3 and 7, and the ammonium/ammonia pair buffers the solution between $^{w}_{w}$pH 7 and 11 approximately. Using the same buffer in all experiments, it is possible to relate the variation of mobile-phase $^{s}_{w}$pH and $^{s}_{s}$pH values during gradient elution with the measured $^{w}_{w}$pH value of the aqueous buffer and therefore to obtain good relationships between retention and $^{w}_{w}$pH.

EXPERIMENTAL SECTION

Apparatus. A Hewlett-Packard 1090 series high-performance liquid chromatograph with a photodiode array detector was used. Data acquisition and processing were performed on a Viglen IBM-compatible PC with HP Chemstation software (Hewlett-Packard Co., Amsterdam, The Netherlands). The reversed-phase HPLC measurements were carried out on a 5-μm C-18 Luna column with the dimensions of 50 × 4.6 mm. pH measurements were taken with a Ross semimicrocombination electrode Orion 8103 (glass electrode and a reference electrode with a 3.0 M KCl solution in water as a salt bridge) in a Radiometer Copenhagen PHM93 reference pH meter with a precision of ±0.1 mV (±0.002 pH unit). All measurements were made in an air-conditioned room with a temperature of 26.0 ± 0.1 °C, as measured by the HP Chemstation.

Chemicals. HPLC grade methanol and acetonitrile were used as organic modifiers. The studied samples were as follows: benzoic acid, 4-*tert*-butylbenzoic acid, aniline, 4-*tert*-butylaniline, 4-*tert*-butylpyridine, papaverine, lidocaine, ephedrine, 4-*tert*-butylbenzylamine, 4-*tert*-butylbenzethylamine, and phentermine, all them of analytical grade. Samples were dissolved at ~1 mg/mL in methanol/50 mM ammonium acetate buffer (50:50 v/v).

Procedure. The mobile-phase flow rate was 2.00 mL/min. The gradient mixing was carried out by a high-pressure gradient mixer built into the HP 1090 and controlled by the HP Chemstation program. For the fast gradient retention time measurements, the following gradient program was applied: 0.0−2.5 min, 0−100% organic modifier; 2.5−3.0 min, 100% organic modifier; 3.0−3.2 min, 100−0% organic modifier; and 3.2−4.0 min, 0% organic modifier.

The aqueous component of the mobile phase was 50 mM ammonium acetate. The pH was adjusted by adding concentrated formic acid or ammonia solutions of analytical grade. Retention times were measured in duplicate, and the average was used for the calculations. Retention time measurements were repeated using several different starting mobile-phase pHs for each model compounds.

To reveal the pH changes during gradient pH measurements were carried out for a set of 50 mM ammonium acetate buffer solutions, adjusted to different $^{w}_{w}$pH values (ranging from 2.7 to 10.0) by addition of concentrated formic acid or ammonia solutions and diluted with different concentrations of organic solvent (acetonitrile or methanol). The $^{s}_{w}$pH values of these mixtures were also measured with the potentiometric system calibrated with aqueous buffers.

RESULTS AND DISCUSSION

Variation of the pK_a of the Compound Eluted with the Mobile-Phase Gradient. The variation of the solvent composition during gradient elution produces changes in the solvation of the compound that affect its acidity. There are three main effects that cause the change of the pK_a values with added organic solvents: variation of the acid/base properties of the solvent, variation of the dielectric constant of the medium, which affects electrostatic interactions between ions, and variation of the specific solute/solvent interactions (e.g., hydrogen bonding). These effects have been previously studied for methanol/water mixtures,[11,12] and since they are similar for compounds with similar properties, it is possible to establish linear relationships between the pK_a values of a particular family of compounds in water ($^{w}_{w}$pK_a) and the pK_a values of the same family in a fixed methanol/water mixture ($^{s}_{w}$pK_a or $^{s}_{w}$pK_a).

$$^{s}_{s}\mathrm{p}K_a = a_{sw}\,{}^{w}_{w}\mathrm{p}K_a + b_s \quad (2)$$

$$^{s}_{w}\mathrm{p}K_a = a_{sw}\,{}^{w}_{w}\mathrm{p}K_a + b_s + \delta \quad (3)$$

Equations have been also developed to relate the slopes (a_s) and intercepts (b_s) of these relationships with the solvent composition. By means of these equations, the pK_a of an acid at any methanol/water composition can be calculated solely from the pK_a of the same acid in water. Figure 1 presents the variation of the pK_a values in methanol/water of the studied test compounds and buffer components (acetic and ammonium acids), calculated from these equations.[11,12] The aqueous pK values ($^{w}_{w}$pK_a) of the studied model compounds were taken from the literature[13−16] or

(11) Rosés, M.; Rived, F.; Bosch, E. *J. Chromatogr., A* **2000**, *867*, 45−56.
(12) Rived, F.; Canals, I.; Bosch, E.; Rosés, M. *Anal. Chim. Acta* **2001**, *439*, 315−333.
(13) Kortüm, G.; Vogel, W.; Andrussow, K. *Dissociation constants of organic acids in aqueous solution*; IUPAC; Butterworths: London, 1961.
(14) Perrin, D. D. *Dissociation constants of organic bases in aqueous solution*; IUPAC; Butterworths: London, 1961.

Table 1. ${}^{s}_{w}pK_a$ values of Compounds in Methanol/Water Calculated through Eqs 2 and 3 with the Parameters Given in Refs 9 and 10

		% MeOH (v/v)							
	${}^{w}_{w}pK_a$[a]	10	20	30	40	50	60	70	80
benzoic acid	4.20[a]	4.41	4.63	4.87	5.13	5.42	5.72	6.03	6.29
4-*tert*-butylbenzoic acid	4.40[a]	4.62	4.85	5.10	5.37	5.67	6.00	6.32	6.60
aniline	4.58[b]	4.51	4.44	4.37	4.31	4.25	4.18	4.07	3.88
acetic acid	4.76[a]	4.97	5.18	5.41	5.66	5.93	6.21	6.47	6.63
4-*tert*-butylaniline	4.95[c]	4.88	4.81	4.74	4.67	4.60	4.54	4.43	4.23
4-*tert*-butylpyridine	5.99[c]	5.75	5.52	5.30	5.11	4.95	4.81	4.67	4.49
papaverine	6.40[c]	6.14	5.89	5.65	5.42	5.21	5.03	4.89	4.84
lidocaine	7.73[e]	7.64	7.55	7.46	7.37	7.30	7.23	7.11	6.92
ammonia	9.24[d]	9.14	9.04	8.94	8.84	8.77	8.69	8.57	8.37
ephedrine	9.56[c]	9.46	9.35	9.25	9.16	9.08	9.00	8.88	8.68
4-*tert*-butylbenzylamine	9.70[e]	9.60	9.49	9.39	9.29	9.21	9.13	9.02	8.82
4-*tert*-butylbenzethylamine	10.08[e]	9.97	9.86	9.76	9.66	9.58	9.50	9.38	9.18
phentermine	10.23[e]	10.12	10.01	9.91	9.81	9.73	9.65	9.53	9.33

[a] From references (a) 12, (b) 15, (c) 13, (d) 14, and (e) 16.

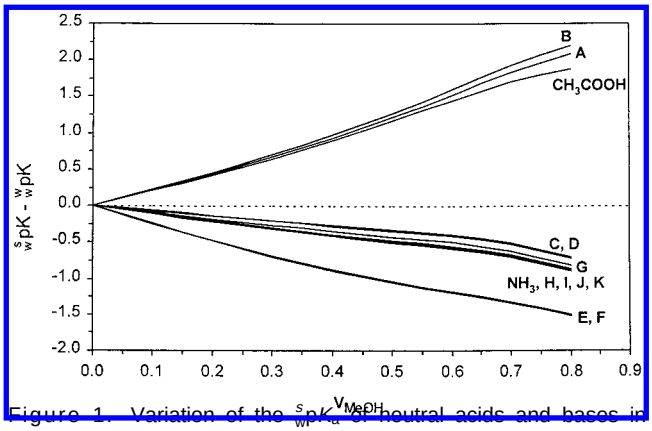

Figure 1. Variation of the ${}^{s}_{w}pK_a$ of neutral acids and bases in methanol/water mixtures. Test compounds: (A) benzoic acid, (B) *tert*-butylbenzoic acid, (C) aniline, (D) 4-*tert*-butylaniline, (E) 4-*tert*-butylpyridine, (F) papaverine, (G) lidocaine, (H) ephedrine, (I) 4-*tert*-butylbenzylamine, (J) 4-*tert*-butylbenzethylamine, and (K) phentermine.

estimated by means of the SPARC program.[17] To maintain consistency with further pH measurements, we have chosen the absolute p*K* scale (${}^{s}_{w}pK_a$) for this plot. The estimation has been limited to the range 0−80% of methanol because for larger methanol contents the originally measured pK_a and δ values are less precise and so is the ${}^{s}_{w}pK_a$ estimation. The calculated pK_a values are also presented in Table 1.

Figure 1 shows that the variation of the acid/base properties of neutral acids and neutral bases is very different. The ${}^{s}_{w}pK_a$ value of neutral acids (benzoic, 4-*tert*-butylbenzoic, and acetic acids) increases with the methanol contents, whereas the ${}^{s}_{w}pK_a$ value of cationic acids obtained by protonation of neutral bases (aniline, 4-*tert*-butylaniline, 4-*tert*-butylpyridine, papaverine, lidocaine, ammonia, ephedrine, 4-*tert*-butylbenzylamine, *tert*-butylbenzethylamine, phentermine) decreases. These differences in acid/base behavior are mostly produced by the difference in the electrostatic interactions of neutral and cationic compounds.[9−12] It can be also observed that the increase in pK_a values of benzoic derivatives is slightly larger than the increase in pK_a values of acetic acid and that the decrease of the pK_a values of 4-*tert*-butylpyridine and papaverine is larger than the decrease in pK_a values of amine derivatives.

Unfortunately, there is not yet enough pK_a data in acetonitrile/water mixtures to establish equations to estimate the pK_a value of compounds for any acetonitrile/water composition. However, the trends observed in the experimental data available[18,19] are similar to those observed for methanol/water. The ${}^{s}_{w}pK_a$ value of neutral acids increases with the acetonitrile contents, with this increase larger for aromatic carboxylic acids than for aliphatic carboxylic acids. The ${}^{s}_{w}pK_a$ values of protonated amines decrease with the acetonitrile content, and the ${}^{s}_{w}pK_a$ values of protonated pyridines decrease even more.

Variation of the pH Value of the Buffer with the Mobile-Phase Gradient. Figure 1 has shown that the pK_a value of acetic and ammonium acids changes with the composition of the mobile phase. Since we have used buffers composed of ammonium acetate, the pH of these buffers is also expected to change with the mobile-phase composition. This change has been measured for several initial aqueous buffers to which methanol or acetonitrile was added. The pH electrode system was calibrated with the usual aqueous buffers of pH 4 and 7, and therefore, the pH readings obtained were in the absolute pH scale (${}^{s}_{w}pH$). The pH values obtained are presented in Tables 2 and 3, and the pH variation is given in Figure 2.

For buffers with initial (aqueous) pH values below 7, the ${}^{s}_{w}pH$ value of the buffer increases when the methanol or acetonitrile content increases, because so does the ${}^{s}_{w}pK_a$ value of the acetic/acetate pair that buffers these solutions. However, for buffers with ${}^{w}_{w}pH$ values above 8, the ${}^{s}_{w}pH$ decreases because these solutions are buffered by the ammonia/ammonium pair and the ${}^{s}_{w}pK_a$ value of this acid/base pair decreases with the addition of methanol or acetonitrile (Figure 1). The buffer with a ${}^{w}_{w}pH$ value of 7.94 presents an intermediate behavior. The ${}^{s}_{w}pH$ value of this

(15) Perrin, D. D. *Dissociation constants of inorganic acids and bases in aqueous solution*; IUPAC; Butterworths: London, 1969.
(16) Perrin, D. D. *Dissociation constants of organic bases in aqueous solution: Supplement*; IUPAC; Butterworths: London, 1972.
(17) Hilal, S. H.; Karickhoff, S. W.; Carreira, L. A. *Quantum Struct.-Act. Relat.* **1995**, *14*, 348−355.
(18) Espinosa, S.; Rosés, M.; Bosch, E., submitted.
(19) Pawlak, Z. *J. Chem. Thermodyn.* **1987**, *19*, 443−447.

Table 2. Measured $^{s}_{w}$pH Values of 50 mM Ammonium Acetate at Different Methanol/Water Compositions

v_{MeOH}	$^{s}_{w}$pH								
0.00	2.67	3.01	4.06	5.07	6.07	6.96	7.94	8.94	9.95
0.10	2.81	3.16	4.18	5.17	6.16	7.01	7.91	8.90	9.93
0.20	2.97	3.27	4.29	5.31	6.29	7.08	7.83	8.82	9.82
0.30	3.11	3.41	4.43	5.47	6.45	7.10	7.74	8.71	9.73
0.40	3.31	3.59	4.61	5.69	6.66	7.20	7.72	8.65	9.68
0.50	3.50	3.78	4.84	5.93	6.88	7.29	7.69	8.57	9.60
0.60	3.73	4.04	5.10	6.21	7.11	7.43	7.71	8.52	9.53
0.70	3.97	4.29	5.40	6.50	7.32	7.55	7.74	8.45	9.44
0.80	4.23	4.58	5.70	6.80	7.50	7.64	7.82	8.38	9.37

Table 3. Measured $^{s}_{w}$pH Values of 50 mM Ammonium Acetate at Different Acetonitrile/Water Compositions

v_{MeCN}	$^{s}_{w}$pH								
0.00	2.67	3.01	4.06	5.07	6.07	6.96	7.94	8.94	9.95
0.10	2.81	3.16	4.22	5.24	6.25	7.07	7.91	8.89	9.89
0.20	2.95	3.30	4.40	5.46	6.45	7.13	7.82	8.81	9.82
0.30	3.13	3.51	4.63	5.71	6.68	7.26	7.78	8.73	9.73
0.40	3.35	3.71	4.86	5.96	6.90	7.35	7.75	8.65	9.65
0.50	3.61	3.96	5.10	6.20	7.10	7.44	7.74	8.57	9.57
0.60	3.81	4.18	5.33	6.46	7.27	7.52	7.73	8.45	9.46
0.70	4.12	4.47	5.64	6.77	7.46	7.61	7.73	8.32	9.29
0.80	4.53	4.91	6.09	7.18	7.64	7.71	7.78	8.14	9.05

buffer shows only a small variation because both acid/base pairs (acetic/acetate, ammonium/ammonia) contribute to buffer the solution and the increase in acetic pK_a value when methanol or acetonitrile percentage increases is more or less balanced by the decrease in ammonium pK_a value. It also means that when we measure the fast gradient retention times starting at pH 7.4 ammonium acetate buffer, the $^{s}_{w}$pH variation is very small (less than 0.2 pH unit) with both methanol and acetonitrile gradients.

Figure 2 shows that the variation of the pH values is approximately proportional to the volume fraction of methanol or acetonitrile in the solvent mixture. This proportionality should allow us to calculate the $^{s}_{w}$pH value of a buffer in any methanol/water or acetonitrile/water mixture from the aqueous pH value of the buffer before mixing it with the organic modifier. The $^{s}_{w}$pH values of the buffers have been thus fitted to the equation

$$^{s}_{w}\mathrm{pH} = {}^{w}_{w}\mathrm{pH} + m v_s \qquad (4)$$

where v_s is the volume fraction of methanol or acetonitrile. The slopes (m) are approximately constant and positive for $^{w}_{w}$pH <6, where the acetic/acetate pair buffers the solution, and constant and negative for $^{w}_{w}$pH >8, where the solution is buffered by the ammonium/ammonia pair. The variation in the slopes for acetonitrile/water mixtures is larger than for methanol/water because methanol is more similar to water than acetonitrile and causes a lower variation of pH and pK values. The sigmoidal plots obtained for the slopes allow to be fitted to equations of the type

$$m = \frac{a10^{(b-{}^{w}_{w}\mathrm{pH})} + c}{10^{(b-{}^{w}_{w}\mathrm{pH})} + 1} \qquad (5)$$

The best fits, which minimize the square residuals between observed and calculated $^{s}_{w}$pH values, have been obtained for a = 1.83, b = 7.14, and c = −0.72 (SD = 0.07, F = 1068) for methanol/water and a = 2.19, b = 7.17, and and c = −0.88 (SD = 0.15, F = 362) for acetonitrile/water. These parameters together with eqs 4 and 5 allow us to calculate the pH variation during gradient elution in the absolute scale of any prepared aqueous ammonium acetate buffer.

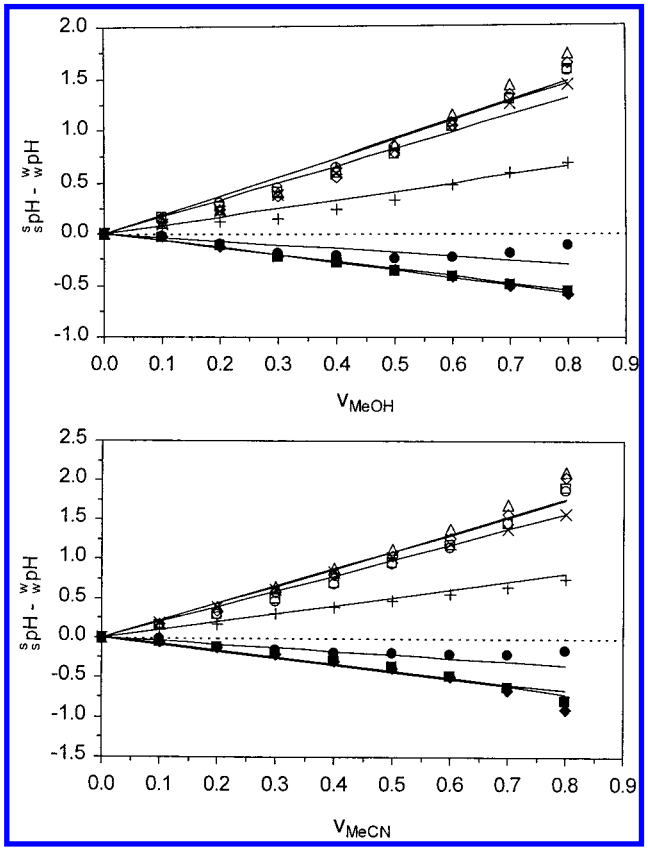

Figure 2. Variation of the $^{s}_{w}$pH of the buffers in methanol/water and acetonitrile/water mixtures. $^{w}_{w}$pH values: (○) 2.67, (□) 3.01, (◇) 4.06, (△) 5.07, (×) 6.07, (+) 6.96, (●) 7.94, (■) 8.94, and (◆) 9.95.

Effect of Mobile-Phase Change in Isocratic Elution. The effect of the mobile-phase change during gradient elution can be studied by isocratic measurements of retention at different mobile-phase compositions and pH values. It is well known that the retention of an acid/base compound with an isocratic mobile phase can be related to the pH of the mobile phase by means of the relationship[9,10,20−24]

$$t_R = (t_{R(HA)}10^{(pK_a'-pH)} + t_{R(A)})/(10^{(pK_a'-pH)} + 1) \qquad (6)$$

where $t_{R(HA)}$ and $t_{R(A)}$ are the retention times of the acidic and basic forms of the compound, respectively, and pK_a' is the pK_a value of the compound in terms of concentrations instead of activities of the acid/base species HA and A. The retention time in eq 6 can

(20) Lopes Marques, R. M.; Schoenmakers, P. J. *J. Chromatogr.* **1992**, *592*, 157−182.
(21) Schoenmakers, P. J.; Tijssen, R. *J. Chromatogr.* **1993**, *656*, 577−590.
(22) Lewis, J. A.; Lommen, D. C.; Raddatz, W. D.; Dolan, J. W.; Snyder, L. R.; Molnár, I. *J. Chromatogr.* **1992**, *592*, 183−195.
(23) Lewis, J. A.; Dolan, J. W.; Snyder, L. R.; Molnár, I. *J. Chromatogr.* **1992**, *592*, 197−208.
(24) Horváth, C.; Melander, W.; Molnár, I. *Anal. Chem.* **1977**, *49*, 142−154.

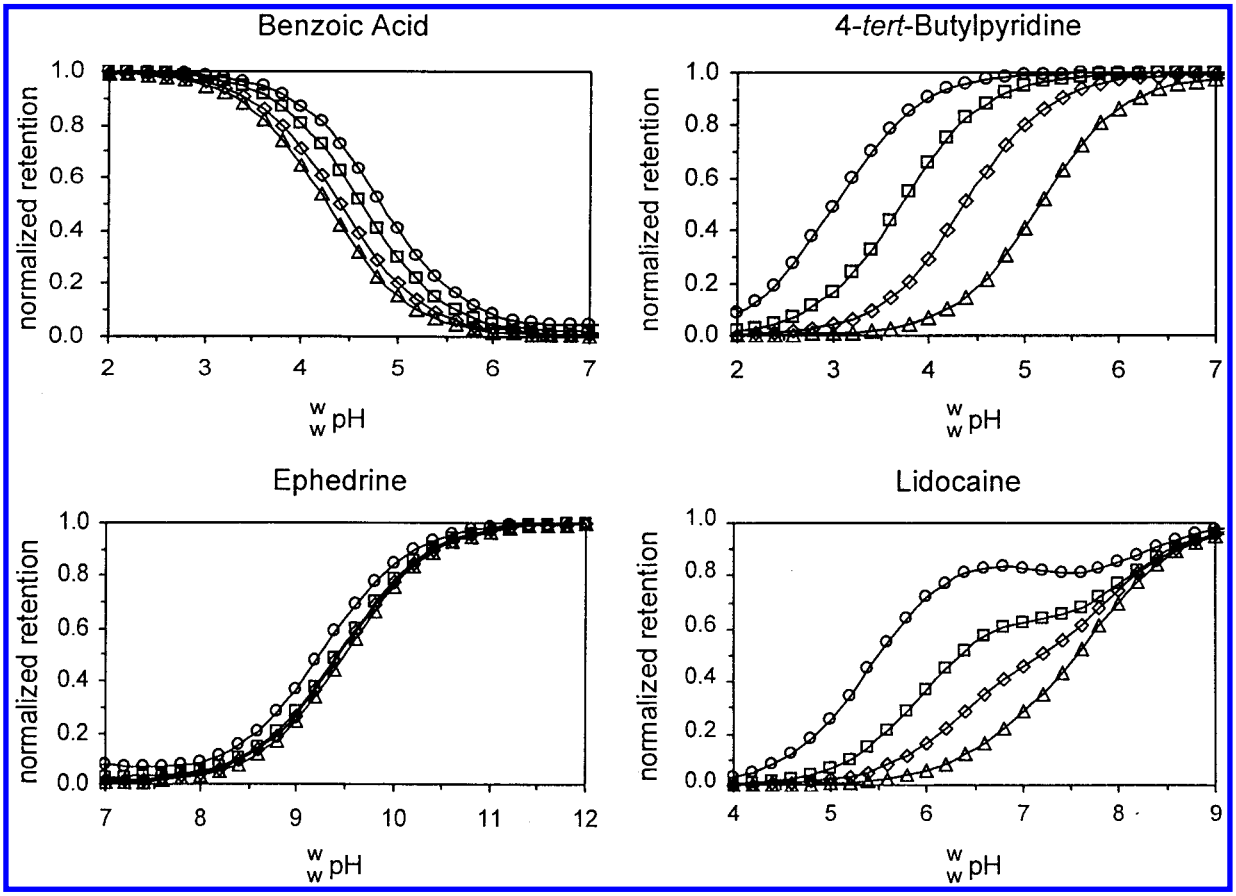

Figure 3. Calculated retention plots for selected compounds in several isocratic methanol/water mobile phases. Methanol concentrations: (△) 20, (◇) 40, (□) 60, and (○) 80%.

be replaced by adjusted retention time or retention factor provided that a constant holdup time is taken. The pH can be given in any of the rigorous pH scales measured in the mobile phase, ${}^{s}_{w}$pH or ${}^{s}_{s}$pH, and the fitting parameter obtained, pK_a, will be also in the same scale used for pH (${}^{s}_{w}$pK_a or ${}^{s}_{s}$pK_a, respectively). Equation 6 does not hold for the ${}^{w}_{w}$pH scale (i.e., the pH measured in the aqueous buffer before mixing it with the organic modifier) unless the same type of buffers is used in the pH range of variation of retention. If the same type of buffers is used for all pH points, the ${}^{s}_{w}$pH or ${}^{s}_{s}$pH scales can be related to ${}^{w}_{w}$pH scale (i.e., eqs 4 and 5 in our case) and relationships of the type such as eq 6 should be obtained between retention and pH.

We have tested these relationships for the studied compounds by calculation of the variation of retention with mobile-phase composition and ${}^{w}_{w}$pH in methanol/water. Since the mobile-phase variation produces changes in $t_{R(HA)}$ and $t_{R(A)}$, in addition to pK_a and pH, the studied parameter has been normalized retention (t^N):

$$t^N = (t_R - t_{R(A)})/(t_{R(HA)} - t_{R(A)}) \qquad (7)$$

which is equivalent to using $t_{R(HA)} = 1$ and $t_{R(A)} = 0$ for a neutral acid or $t_{R(HA)} = 0$ and $t_{R(A)} = 1$ for a neutral base in eq 6. The retention of the different compounds studied has been calculated for several methanol/water mobile phases by using these $t_{R(HA)}$ and $t_{R(A)}$ values and the ${}^{s}_{w}$pK_a of each compound at each mobile-phase composition as pK'_a(taken from Table 1). ${}^{w}_{w}$pH values from 2 to 12, at 0.2 pH unit intervals, were used to calculate the corresponding ${}^{s}_{w}$pH values for each mobile-phase composition from eqs 4 and 5, and these ${}^{s}_{w}$pH values were taken as pH values in eq 6 to compute t_R (normalized retention). The obtained retention times were plotted against the initial ${}^{w}_{w}$pH values. Figure 3 presents the obtained results for four representative compounds.

The shifts with the mobile-phase variation in the t^N versus ${}^{w}_{w}$pH plots are quite different. When the methanol proportion in the mobile phase increases, there is a slight shift in the plot for benzoic acid toward larger ${}^{w}_{w}$pH values. This shift can be explained because the pH range of variation of benzoic retention is covered by acetic/acetate buffers. The ${}^{s}_{w}$pK_a of acetic acid increases with the methanol contents (see Table 1 and Figure 1) and so do the ${}^{s}_{w}$pH values of these buffers (Table 2 and Figure 2). Nevertheless, the ${}^{s}_{w}$pK_a value of benzoic acid also increases with the methanol percentage, but in a slightly larger degree than for acetic acid (Table 1 and Figure 1). Therefore, the relative differences ${}^{s}_{w}$pK_a − ${}^{s}_{w}$pH for a specific initial ${}^{w}_{w}$pH value increase when the methanol contents increase, and the inflection point of the plots (which agree with the pK'_a parameter of eq 6) moves toward larger ${}^{w}_{w}$pH values.

Acetic/acetate pair also buffers the pH range for variation of retention of *tert*-butylpyridine, but when the methanol percentage increases, the ${}^{s}_{w}$pK_a values of *tert*-butylpyridinium decrease. The combination in the increase of the ${}^{s}_{w}$pH values of the buffer and the decrease in the ${}^{s}_{w}$pK_a value causes a large decrease of the ${}^{s}_{w}$pK_a − ${}^{s}_{w}$pH differences and an important shift of the plots

toward lower ${}^{w}_{w}$pH values when the methanol percentage in the mobile phase increases.

In contrast, the ephedrine pH range is buffered by the ammonium/ammonia pair. The ${}^{s}_{w}pK_a$ values of both amines (ephedrine, ammonia) decrease in a very similar degree with the increase in the methanol percentage (Figure 1, Table 1). Therefore, the ${}^{s}_{w}pK_a - {}^{s}_{w}$pH differences for the same initial ${}^{w}_{w}$pH value remain quite constant with the mobile-phase change and the plots almost do not shift. Only a very slight shift toward lower ${}^{w}_{w}$pH values is observed when the methanol contents increase, which indicates a slightly larger decrease for ${}^{s}_{w}pK_a$ than for ${}^{s}_{w}$pH (compare variations in Table 1 for ephedrine and Table 2 for the most basic ${}^{w}_{w}$pH values).

Lidocaine has ${}^{s}_{w}pK_a$ values approximately intermediate between those of acetic and ammonium acids, and thus, both acid/base pairs may contribute to buffer the pH range of variation of lidocaine retention. The ${}^{s}_{w}pK_a$ values of lidocaine decrease with the methanol percentage in a way similar to the p*K* values of the other amines.

For small methanol contents (up to ~25%), the ${}^{s}_{w}pK_a$ values of lidocaine are above 7.5. Figure 2 shows that buffers with ${}^{w}_{w}$pH values above 7.5 have a negative slope; i.e., the ${}^{s}_{w}$pH values of these buffers decrease with the methanol contents because they are mostly buffered by ammonium/ammonia. Therefore, for small methanol percentages, lidocaine ${}^{s}_{w}pK_a$ and buffer ${}^{s}_{w}$pH values decrease, both with the methanol percentage increase, and there is only a small shift of the plots of Figure 3 toward lower ${}^{w}_{w}$pH values (the decrease in lidocaine p*K* is slightly larger than in buffer pH, which is close to zero). However, for larger methanol contents, the ${}^{s}_{w}pK_a$ of lidocaine is below 7.5 and buffering solutions with ${}^{w}_{w}$pH values below 7.5 are mostly buffered by acetic/acetate and have positive slopes in Figure 3 (e.g., for 80% methanol where ${}^{s}_{w}pK_a = 6.92$, ${}^{s}_{w}pH = 6.92$ is obtained from a buffer with ${}^{w}_{w}$pH close to 5.2; see Table 2). Therefore, for these methanol percentages the ${}^{s}_{w}pK_a$ values of lidocaine decrease with the increase in methanol contents, but the ${}^{s}_{w}$pH values of the buffers increase. In consequence, there is a large shift of the plots in Figure 3 toward lower ${}^{w}_{w}$pH values with the increase in methanol percentage. Figure 3 shows that the shifts of the curves of lidocaine from 40 to 60% are larger than from 20 to 40% but smaller than from 60 to 80%, whereas the shifts for *tert*-butylpyridine with methanol percentage are rather constant. The plots for high methanol contents also show some distortion in the ${}^{w}_{w}$pH range from 7 to 8 because of the different pH variation of the effective buffer pair below and above pH 7.5.

The shifts of the plots of Figure 3 with the methanol percentage of the mobile phase depend on the relative differences between the variations of the ${}^{s}_{w}pK_a$ values of the compound and the ${}^{s}_{w}$pH values of the buffer. Figure 4 plots these differences for all the compounds studied. As reference ${}^{w}_{w}$pH value of the buffer to calculate the pH variations, we have taken the ${}^{w}_{w}pK_a$ value of the compound. This corresponds to the ${}^{w}_{w}$pH for which the compound is 50% ionized in pure water, i.e., to the inflection point of the retention versus pH plot.

According to Figure 4, shifts in the retention versus ${}^{w}_{w}$pH plots with the increase in methanol contents of the mobile phase are expected for all compounds studied. The shifts should be toward higher ${}^{w}_{w}$pH values for the neutral acids (benzoic and *tert*-butylbenzoic acid) and toward lower ${}^{w}_{w}$pH values for the neutral bases studied. Small shifts are expected for ephedrine, *tert*-butylbenzylamine, *tert*-butylbenzethylamine, and phentermine, slightly larger for lidocaine, quite large for aniline and *tert*-butylaniline, and even larger for papaverine and *tert*-butylpyridine. Figure 4 shows the pK_a shift of the compounds in comparison with the pH shift of the mobile phase caused by increasing methanol concentration. The pK_a values of strong basic compounds that have pK_a values above or around the ammonium hydroxide pK_a shift with increasing methanol concentration in the same direction and to a similar extent as the mobile-phase pH shifts. However, when the pK_a of a basic compound (for example, aniline and 4-*tert*-butylaniline) is in the lower pH region, where the acetate/acetic acid controls the pH, the pH shift of the buffer and the pK_a shift of the compound is in the opposite direction. The figure only reflects well the shifts expected for the compounds whose pH range for variation of retention is buffered always by the same buffer pair, which is acetic/acetate for aniline, 4-*tert*-butylaniline, 4-*tert*-butylpyridine, papaverine, and benzoic and 4-*tert*-butylbenzoic acids and ammonium/ammonia for ephedrine, 4-*tert*-butylbenzylamine, *tert*-butylbenzethylamine, and phentermine. As already explained, lidocaine is buffered by ammonium/ammonia in low methanol percentages and acetic/acetate in high methanol percentages, and in the latter instance, the shifts are larger than expected from Figure 4, which has been calculated for ${}^{w}_{w}$pH = ${}^{w}_{w}pK_a$, i.e., for methanol percentages that tend to zero. These conclusions are expected to hold also for gradient elution because the mobile-phase composition changes with time in this type of elution.

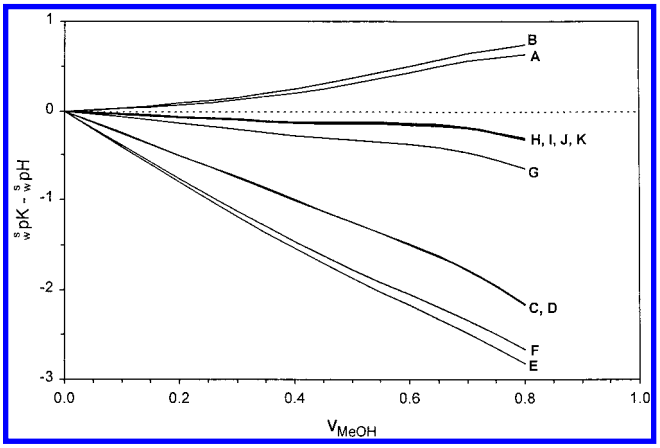

Figure 4. Differences between the variation of the ${}^{s}_{w}pK_a$ value of the compounds and the ${}^{s}_{w}$pH value of a buffer with the same pH in pure water than the aqueous pK_a of the compound. Compounds as in Figure 1.

Effect of Mobile-Phase Change in Gradient Elution. During gradient elution, the methanol or acetonitrile percentage in the mobile-phase changes with time. Therefore, the retention of an analyte is expected to approximately fulfill eq 6 with $t_{R(HA)}$, $t_{R(A)}$, and ${}^{s}_{w}pK_a - {}^{s}_{w}$pH differences that change with time. In a first approximation, one would expect the retention versus ${}^{w}_{w}$pH plot to be an average of the retention versus ${}^{w}_{w}$pH plots obtained for the different mobile-phase percentages (Figure 3).

We have tested this hypothesis by fitting the retention times obtained in gradient elution to the ${}^{w}_{w}$pH of the mobile phase by eq 6. The fitting parameters obtained are presented in Table 4 and

Table 4. Retention Parameters for Test Solutes Using Eq 6

		methanol/water						acetonitrile/water					
	${}^{w}_{w}pK$	pK	$t_{R(HA)}$	$t_{R(A)}$	F	SD	ΔpK	pK	$t_{R(HA)}$	$t_{R(A)}$	F	SD	ΔpK
benzoic acid	4.20	4.95	2.41	1.42	643	0.04	0.75	5.00	1.94	1.24	1135	0.02	0.80
tert-butylbenzoic acid	4.39	4.99	3.02	2.63	583	0.01	0.60	5.32	2.51	1.83	429	0.04	0.93
aniline	4.58	4.03	0.93	2.00	1682	0.02	−0.55	3.96	0.88	1.87	2745	0.02	−0.62
4-*tert*-butylaniline	4.95	3.34	2.25	2.91	414	0.02	−1.61	3.08	1.59	2.58	1898	0.01	−1.87
4-*tert*-butylpyridine	5.99	3.71	1.57	2.86	206	0.07	−2.28	4.12	1.43	2.41	706	0.03	−1.87
papaverine	6.50	4.32	2.13	2.75	2432	0.01	−2.18	4.63	1.78	2.27	2388	0.01	−1.87
lidocaine	7.73	5.58	2.00	2.90	1675	0.02	−2.15	6.00	1.63	2.40	15154	0.00	−1.73
ephedrine	9.56	9.10	1.90	2.41	396	0.02	−0.46	8.62	1.50	1.88	360	0.02	−0.94
4-*tert*-butylbenzylamine	9.70	8.89	2.59	2.99	988	0.01	−0.81	8.57	1.86	2.48	836	0.02	−1.13
4-*tert*-butylbenzethylamine	10.08	9.09	2.72	3.10	479	0.01	−0.99	8.81	1.96	2.62	586	0.03	−1.27
phentermine	10.23	9.23	2.18	2.78	566	0.02	−1.00	9.06	1.64	2.23	259	0.03	−1.17

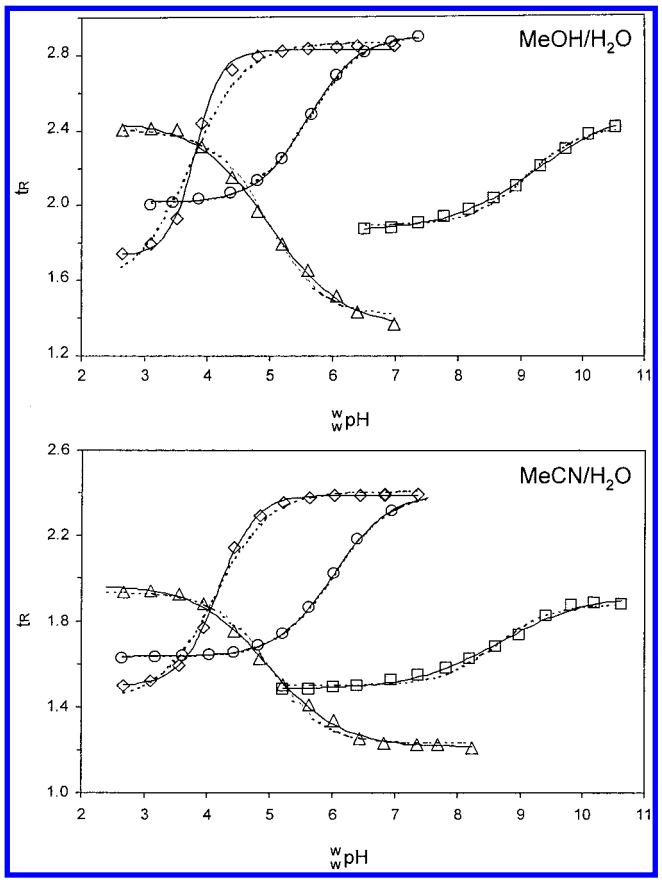

Figure 5. Retention of the compounds of Figure 3 in gradient elution with methanol/water and acetonitrile/water mobile phases. Dashed line fitted by eq 6. Continuous line fitted by eq 8. Compounds: (△) benzoic acid, (◇) 4-*tert*-butylpyridine, (□) ephedrine, and (○) lidocaine.

some representative plots in Figure 5. As expected, the retention times of neutral forms of the compounds studied are larger than the retention times of the ionized forms, and the fitting pK'_a values do not agree with the pK_a values of the compounds in water (${}^{w}_{w}pK_a$). However, the differences between both pK_a values agree quite well with the predictions from the isocratic study. With methanol/water, the neutral acids (benzoic and *tert*-butylbenzoic) give pK'_a values higher than their pK_a in water, whereas the neutral bases give lower pK'_a values. The largest differences (ΔpK) are obtained for *tert*-butylpyridine, papaverine, lidocaine, and *tert*-butylaniline. The lowest differences observed for bases are for phentermine, *tert*-butylbenzethylamine, *tert*-butylbenzylamine, aniline, and ephedrine. According to Figure 4, the pK difference for aniline should be close to that for *tert*-butylaniline. The reason for the small pK variation for aniline is that this compound is much less retained than the others, and therefore, its ${}^{s}_{w}pK_a$ and the buffer ${}^{s}_{w}$pH variation during elution is much lower than for the other compounds. Although not exactly equal, the results obtained for the acetonitrile/water gradient elution follow the same trends as for methanol/water.

Although the fits obtained with eq 6 are quite good, they can be improved by consideration of the dynamics of gradient elution. When a compound is chromatographed with gradient elution, the mobile phase changes between the initial composition and the particular composition obtained at the elution time of the compound. This means that the mobile phase changes less for short elution times than for large elution times. Therefore, the compound ${}^{s}_{w}pK_a$ and the buffer ${}^{s}_{w}$pH variations for the same compound are smaller in pH buffers that give short retention times than in buffers that produce large retention. This causes a distortion on the t_R versus ${}^{w}_{w}$pH plots because points with large retention are shifted more than points with short retention. The distortion can be measured by an additional parameter, s, added to eq 6 which enlarges the pK'_a − pH difference to retention. The equation takes the form

$$t_R = (t_{R(HA)}10^{s(pK_a'-pH)} + t_{R(A)})/(10^{s(pK_a'-pH)} + 1) \quad (8)$$

The results obtained with this equation are presented in Table 5. The statistical parameters of Table 5 are in general better than those of Table 4. Some compounds have an s parameter close to 1, and the statistics for these compounds with eq 8 are worse than with eq 6 because the fit with both equations is very similar but there is one additional fitting parameter in eq 8.

The interpretation of the values of the s parameter is complex and difficult, although it is clear that it depends on two main factors. One is the retention times of the ionized and un-ionized forms of the compounds, which determine the mobile-phase composition at which these forms are eluted. The other factor is the compound ${}^{s}_{w}pK_a$ and the buffer ${}^{s}_{w}$pH variation between these two mobile-phase compositions. Most neutral forms of the compounds are eluted at retention times close to or even larger than 2.5 min, and for them the mobile-phase composition is 100% organic solvent or close to this value. The ${}^{s}_{w}pK_a$ variation in this zone (80−100% organic solvent) is difficult to precisely ascertain, although all compounds, including neutral bases, present a large

Table 5. Retention Parameters for Test Solutes Using Eq 8

		methanol/water							acetonitrile/water							
	${}^{w}_{w}pK$	pK	$t_{R(HA)}$	$t_{R(A)}$	s	F	SD	ΔpK	Δt_R	pK	$t_{R(HA)}$	$t_{R(A)}$	s	F	SD	ΔpK	Δt_R
benzoic acid	4.20	4.99	2.45	1.35	0.75	1171	0.02	0.79	−1.10	4.98	1.97	1.21	0.76	2356	0.01	0.78	−0.76
tert-butylbenzoic acid	4.39	5.03	3.04	2.61	0.74	833	0.01	0.64	−0.43	5.36	2.56	1.77	0.62	983	0.02	0.97	−0.79
aniline	4.58	4.01	0.92	2.00	0.96	1031	0.02	−0.57	1.08	3.97	0.90	1.87	1.05	1729	0.02	−0.61	0.97
4-*tert*-butylaniline	4.95	3.50	2.36	2.90	1.60	1074	0.01	−1.45	0.54	3.25	1.76	2.57	1.31	16122	0.00	−1.70	0.81
4-*tert*-butylpyridine	5.99	3.80	1.74	2.82	2.08	903	0.03	−2.19	1.08	4.17	1.50	2.39	1.47	5121	0.01	−1.82	0.89
papaverine	6.50	4.33	2.14	2.74	1.08	1690	0.01	−2.17	0.60	4.63	1.79	2.26	1.18	6247	0.01	−1.87	0.47
lidocaine	7.73	5.58	2.00	2.91	1.00	993	0.02	−2.15	0.91	6.01	1.63	2.40	0.98	9355	0.01	−1.72	0.77
ephedrine	9.56	9.23	1.86	2.50	0.63	1586	0.01	−0.33	0.64	8.68	1.48	1.92	0.64	616	0.01	−0.88	0.44
4-*tert*-butylbenzylamine	9.70	8.92	2.58	3.02	0.78	2153	0.01	−0.78	0.44	8.61	1.84	2.52	0.73	2184	0.01	−1.09	0.68
4-*tert*-butylbenzethylamine	10.08	9.20	2.70	3.15	0.67	1673	0.01	−0.89	0.45	8.91	1.93	2.69	0.66	2290	0.01	−1.17	0.76
phentermine	10.23	9.38	2.14	2.89	0.66	3466	0.01	−0.85	0.75	9.15	1.60	2.32	0.63	377	0.02	−1.08	0.72

increase in their ${}^{s}_{w}pK_a$ values because they become desolvated by water and fully solvated by the less polar solvent methanol or acetonitrile. The buffer ${}^{s}_{w}$pH variation is even more difficult to ascertain because the mobile phase is prepared by mixing the aqueous buffer with the pure organic modifier, and thus, the 100% methanol or acetonitrile mobile phase is not buffered.

Nevertheless, some common trends for compounds of the same type, eluted with the same type of buffer, can be observed in the values of Table 5. Benzoic and *tert*-butylbenzoic acids show *s* values close to 0.7, likely because of the slightly larger variation of the ${}^{s}_{w}pK_a$ of these acids in reference to the variation of the ${}^{s}_{w}pK_a$ of acetic acid (Figure 1) and thus the ${}^{s}_{w}$pH variation of the buffer. A similar behavior is observed for ephedrine, *tert*-butylbenzylamine, *tert*-butylbenzethylamine, and phentermine, buffered by ammonium/ammonia buffers, which ${}^{s}_{w}pK_a$ values at high organic solvent composition are slightly larger than that of ammonia (Figure 1).

tert-Butylpyridine, *tert*-butylaniline, and to a minor degree papaverine have *s* values larger than 1 because they have ${}^{s}_{w}pK_a$ values much lower than those of acetic acid, which buffers the pH range of variation of retention of these compounds.

We do not have a clear explanation for the *s* value of lidocaine, which is very close to 1, although it may be related to the small variation of the buffer ${}^{s}_{w}$pH values in the range of variation of retention of this compound, buffered by both ammonium/ammonia and acetic/acetate solutions.

Aniline is the less retained of the studied compounds, and variation between the mobile-phase compositions at which its two forms are eluted is smaller than for the other compounds. Therefore, the distortion of the plots is small and *s* is close to 1.

The *s* and ΔpK values of Table 5 show some common dependence. The *s* values of the two benzoic acids are very similar, and so are the two ΔpK values. For the neutral bases, an increase in the *s* value is accompanied by a decrease of the ΔpK value, because both parameters are related to the pH/pK variation during gradient elution. However, it is important to clarify the exact meaning of each parameter. ΔpK measures the shift of the inflection point of the t_R versus ${}^{s}_{s}$pH plot (pK'_a in eq 8) in reference to the ${}^{w}_{w}pK_a$ value of the compound (pK_a in pure water), whereas *s* is related to the slope (first derivative) of the plot in this inflection point, which depends on the differences in the shifts of the points before and after this inflection point. The position of the inflection point of the retention versus pH plots depends on the relative variation of the pK_a of the compound during the elution

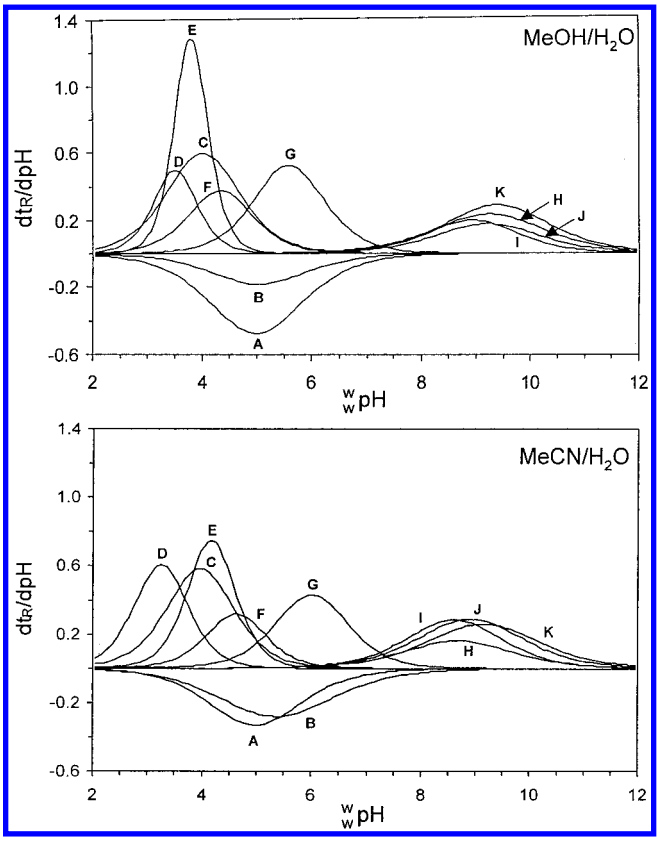

Figure 6. First derivative of the retention time of the studied compounds with pH. Compounds as in Figure 1.

in reference to the variation of the pH values of the buffer. This can be observed in Figure 6, where the first derivative of the retention time has been plotted. One peak is obtained for each compound, positive for the neutral bases and negative for the neutral acids, because the area of the peak is the $t_{R(A)} - t_{R(HA)}$ difference. The ${}^{w}_{w}pK_a$ and ΔpK values of the compound determine the position of the maximum of the peak in the pH scale, which equals the pK'_a value of eq 8. However, the *s* value (and also the $t_{R(A)} - t_{R(HA)}$ difference) determines the height of the peak since when pH = pK'_a the first derivative becomes

$$dt_R/dpH = 0.70s(t_{R(A)} - t_{R(HA)}) \quad (9)$$

CONCLUSIONS

The change of mobile-phase composition during gradient elution produces changes in the pK_a values of the retained acid/

base compounds and in the pH values of the mobile phase. Because of these changes, the retention of acid/base compounds cannot be related to the pH measured in the mobile phase ($^{s}_{w}$pH and $^{s}_{s}$pH scales). However, good relationships can be obtained with the pH measured in the aqueous buffer not mixed with the organic modifier ($^{w}_{w}$pH) if the same type of buffer is used throughout all experiments.

The retention versus pH plots obtained in gradient elution can be considered an average of the plots obtained in isocratic conditions for all the mobile phases experienced by the chromatographed compound between injection and compound elution. The shape of the plots is similar to those obtained for isocratic elution, although in rigor an additional parameter has to be introduced in the fitting equation to account for the different mobile-phase variation of the less retained (ionized) and more retained (un-ionized) forms of the compound.

The improved equation provides a model to be used in the determination of drug descriptors by fast gradient HPLC and in the optimization of the separations of acid−base compounds.

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