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ARTICLE *in* ANALYTICAL CHEMISTRY · MARCH 2010

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pH Gradient as a Tool for the Separation of Ionizable Analytes in Reversed-Phase High-Performance Chromatography

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The aim of this work was to propose a general scheme of optimizing separation of ionizable analytes and to determine conditions of maximal peak compression in pH-gradient reversed-phase high-performance liquid chromatography (RP HPLC). The approximated explicit equation of the linear pH gradient has been developed. It allows predicting retention times for a given organic modifier content, initial value of pH, and the start and steepness of the pH gradient. Also the formula for calculating maximal peak compression is provided. The developed theory was compared with experimental data on the example of a weak acid (ketoprofen) and a weak base (papaverine). Five parameters characterizing analyte retention (log k_w and S of the ionized and nonionized forms along with p$K_{a,chrom}$) were determined in a series of isocratic experiments carried out at different pH values and with different methanol contents in the eluent. Next, a series of pH gradients of different pH-gradient steepness and of different pH-gradient starting time has been obtained and used to test the validity of our theoretical approach. The conditions of maximal peak width compression have been found. The derived theory was proved to be in a good agreement with the experimental data. The pH-gradient method led to peak compression of up to 0.2, and minimized peak tailing was obtained for the tested analytes. Since the majority of analytical separations are done in an isocratic mode we proposed a means to transfer an isocratic method to a pH-gradient method.

The pH gradient is a new separation technique applicable to ionogenic analytes in reversed-phase high-performance liquid chromatography (RP HPLC). It consists on the programmed linear or nonlinear changes of the mobile phase pH with constant organic modifier content during the chromatographic separation. The increase of pH for acids and decrease of pH for bases affect the degree of analyte dissociation and lead to the changes in analyte retention. The pH gradient has typical features of the gradient RP HPLC, like peak compression, improved peak sensitivity, and minimized peak tailing. However, the degree of peak compression highly depends on the parameters of the pH gradient, like gradient steepness, and is more difficult to describe theoretically than organic modifier gradient.[1−3] A narrow pH gradient was first described and used in RP HPLC in 1991.[4] Since then attempts have been undertaken to describe the pH-gradient mode theoretically and to use it for pK_a determination of monoprotic acids and bases.[5,6] Unfortunately, the applicability of the pH gradient remains limited probably due to the lack of a simple, explicit model enabling predictions of retention times for a specified analyte and linear pH gradient. In this work we would like to provide a theoretical approach that will provide the appropriate formulas. We will also look for conditions leading to the high peak compression. The accuracy of the proposed theory will be illustrated by the experimental data for ketoprofen and papaverine at various pH gradients.

THEORETICAL SECTION

The general equation describing the dependence of the analyte retention during isocratic and gradient conditions has the form[7,8]

$$\int_0^{t'_R} \frac{dt}{t_0 k_i(t)} = 1 \qquad (1)$$

where $k_i(t)$ is the instantaneous retention factor referring to the isocratic retention factor, k, which would be obtained with the mobile phase composition actually present at a column inlet, t_0 is a hold-up time, $t'_R = t_R - t_0 - t_e$ represents an adjusted retention time, and t_e is an extracolumn time. The practical usage of eq 1 is feasible when $k_i(t)$ is known for each composition of the mobile phase occurring during gradient. In particular the combined pH/organic modifier gradient model has been proposed to describe the effect of both pH and organic modifier content on the monoprotic analyte retention:[9]

$$k_i(t) = \frac{10^{\log k_{w,1} - S_1\varphi(t)} + 10^{\log k_{w,2} - S_2\varphi(t)} 10^{\mathrm{pH}(t) - \mathrm{p}K_{a,chrom}}}{1 + 10^{\mathrm{pH}(t) - \mathrm{p}K_{a,chrom}}} \qquad (2)$$

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10.1021/ac100023z
Published on Web 03/30/2010

where parameter $k_{w,j}$ ($j = 1, 2$) denotes the retention factor in a neat water eluent of the ionized or nonionized form of an analyte. For bases $k_{w,1} < k_{w,2}$; thus, $k_{w,1}$ refers to the ionized form and $k_{w,2}$ to the nonionized form of the analyte (the retention factor of the ionized form is always lower than that for the nonionized form). In the case of acids $k_{w,1} > k_{w,2}$; thus, $k_{w,1}$ refers to the nonionized form and $k_{w,2}$ to the ionized form of the analyte. S_j are constants, characteristic for each analyte form and the chromatographic system involved. The $pK_{a,chrom}$ is a zeroth-order approximation to the true relationship between pK_a and φ. It is also a measure of ionization of an analyte and can be viewed as a resultant of all the pK_a values occurring during the chromatographic run as a consequence of changes in the organic modifier content.[9] $\varphi(t)$ and pH(t) indicate changes of organic modifier and pH at the column inlet and depend on the applied pump program. For other possible relationship see the reviews.[10,11]

At isocratic conditions the retention time is given by

$$t_R = t_0 \frac{10^{\log k_{w,1} - S_1\varphi} + 10^{\log k_{w,2} - S_2\varphi} 10^{pH - pK_{a,chrom}}}{1 + 10^{pH - pK_{a,chrom}}} + t_0 + t_e \quad (3)$$

Generally, eq 1, when combined with eq 2, has been shown to be a very good mathematical model to describe the retention data during the organic modifier gradient for different pH changes of the mobile phase.[9,12−14] In this work we would like to prove that it is also applicable for the prediction of retention in the pH gradient.

pH Gradient. During pH gradient the organic modifier content is constant:

$$\varphi(t) = \varphi_x \quad (4)$$

and pH changes continuously. For that situation, eq 1 combined with eq 2 gives

$$\int_0^{t_R'} \frac{1}{t_0} \frac{1 + 10^{pH(t) - pK_{a,chrom}}}{10^{\log k_{w,1} - S_1\varphi_x} + 10^{\log k_{w,2} - S_2\varphi_x} 10^{pH(t) - pK_{a,chrom}}} dt = 1 \quad (5)$$

Equation 5 can be used to predict pH-gradient retention times whenever the parameters characterizing analyte retention ($k_{w,1}$, $k_{w,2}$, S_1, S_2, and $pK_{a,chrom}$) are known. However, it cannot be solved explicitly for the majority of pH(t) changes.

The Explicit Solution. The approximate solution of eq 5 can be proposed for a linear form of the pH gradient:

$$pH(t) = \begin{cases} pH_0 & \text{for } t \le t_d + t_s \\ pH_0 + a(t - t_d - t_s) & \text{for } t_d + t_s < t \le t_G + t_d + t_s \\ pH_f & \text{for } t > t_G + t_d + t_s \end{cases} \quad (6)$$

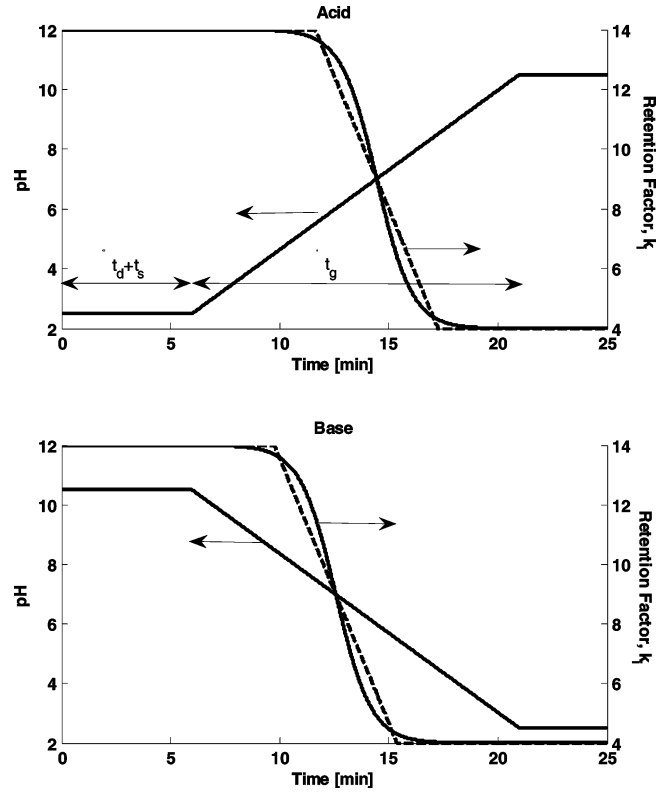

Figure 1. Schematic representation of the pH changes during the pH gradient and corresponding changes of k_i for monoprotic acid and base given by the true sigmoidal relationship and its piecewise continuous approximation (eq 9).

where t_G is the gradient duration, a is the steepness of the gradient $a = (pH_f - pH_0)/t_G$, t_d denotes dwell time (time that gradient needs to begin at the column inlet), and t_s is the pH-gradient starting time (time the pH gradient starts in the pump program). Further, it is assumed that both acidic and basic analytes are totally nonionized at the beginning of the gradient and fully ionized at the end of the gradient:

$$\begin{aligned} i(pH_0 - pK_{a,chrom}) &\le -1.5 \\ i(pH_f - pK_{a,chrom}) &\ge 1.5 \end{aligned} \quad (7)$$

where i is an indicator variable:

$$i = \begin{cases} 1 & \text{for acids} \\ -1 & \text{for bases} \end{cases} \quad (8)$$

From eq 7 it is clear that for acids pH has to increase ($a > 0$), whereas for bases it has to decrease ($a < 0$) with time. Other situations are of minor practical interest and for the sake of simplicity are omitted in these considerations. Under the above given assumptions the $k_i(t)$ can be accurately simplified by a set of piecewise linear functions with the retention factor changing linearly in the pH range equal to $pK_a \pm 1.5$ and being constant otherwise. The range of $pK_a \pm 1.5$ was selected to ensure that analyte is fully dissociated or nondissociated within those values. The proposed simplification of $k_i(t)$ is presented in Figure 1. Mathematically it is described by the set of following equations:

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$$k_i(t) = \begin{cases} k_{\text{nion}} & \text{for} \quad t \le t_1 \\ k_{\text{nion}} - i\dfrac{k_{\text{nion}} - k_{\text{ion}}}{3} a(t - t_1) & \text{for} \quad t_1 < t \le t_2 \\ k_{\text{ion}} & \text{for} \quad t > t_2 \end{cases} \tag{9}$$

where t_1 and t_2 are times defining the time frame when the pH changes affect analyte retention:

$$\begin{aligned} t_1 &= t_{\text{d}} + t_{\text{s}} + \frac{\text{p}K_{\text{a,chrom}} - i1.5 - \text{pH}_0}{a} \\ t_2 &= t_{\text{d}} + t_{\text{s}} + \frac{\text{p}K_{\text{a,chrom}} + i1.5 - \text{pH}_0}{a} \end{aligned} \tag{10}$$

and k_{ion}, k_{nion} are the retention factors of the ionized and the nonionized forms of the analyte, respectively, for different organic modifier contents in the eluent:

$$\begin{aligned} k_{\text{nion}} &= \max(k_{\text{w},1}10^{-S_1\varphi_x}, k_{\text{w},2}10^{-S_2\varphi_x}) \\ k_{\text{ion}} &= \min(k_{\text{w},1}10^{-S_1\varphi_x}, k_{\text{w},2}10^{-S_2\varphi_x}) \end{aligned} \tag{11}$$

For $k_i(t)$ given by eq 9 the solution of eq 1 is feasible and leads to

$$t'_{\text{R}} = \begin{cases} t_{\text{o}}k_{\text{nion}} & \text{for} \quad t'_{\text{R}} < t_1 \\ t_1 + \dfrac{3k_{\text{nion}}}{ai(k_{\text{nion}} - k_{\text{ion}})}\left(1 - \text{e}^{-(1-[t_1/(t_0k_{\text{nion}})]t_0ai[(k_{\text{nion}}-k_{\text{ion}})/3]}\right) & \text{for} \quad t_1 \le t'_{\text{R}} < t_2 \\ t_2 + t_{\text{o}}k_{\text{ion}} - t_1\dfrac{k_{\text{ion}}}{k_{\text{nion}}} + \dfrac{3k_{\text{ion}}}{ai(k_{\text{nion}} - k_{\text{ion}})}\ln\left(\dfrac{k_{\text{nion}}}{k_{\text{ion}}}\right) & \text{for} \quad t_2 \le t'_{\text{R}} \end{cases} \tag{12}$$

Equation 12 allows predicting analyte retention in pH-gradient RP HPLC whenever parameters describing the analyte are known. There are two approaches to utilize eq 12. First, one can determine the retention factors of the ionized and the nonionized form along with the $\text{p}K_{\text{a,chrom}}$ of the analyte for given organic modifier content. Alternatively, one can use eq 2 to determine retention factors of the ionized and nonionized analyte forms in neat water, the S_1 and S_2 parameters reflecting changes of retention factor with organic modifier and the $\text{p}K_{\text{a,chrom}}$. The latter approach is more general, since one can also optimize pH-gradient separation by modifying the organic modifier content in the mobile phase.

Peak Compression. In the case of a linear pH gradient, the analyte migration in the chromatographic column occurs in both the apparent isocratic (for $t < t_1$ and $t > t_2$) and the pH-gradient (for $t > t_1$ and $t < t_2$) conditions. The latter is the phase when the peak compression phenomenon manifests itself. Thus, the smallest peak width should be expected when the retention time after time t_2 is minimized. The retention time for $t < t_1$ is of lesser importance. All that leads to the expectation that the maximal peak compression will occur when the adjusted retention time equals t_2:

$$t'_{\text{R}} = t_2 \tag{13}$$

Combining eqs 12 and 13 leads to the optimal (giving the maximal peak compression) value of a_{opt}:

$$a_{\text{opt}} = \frac{k_{\text{nion}}}{k_{\text{nion}}t_{\text{o}} - t_{\text{d}} - t_{\text{s}}}\left(\frac{\text{p}K_{\text{a,chrom}} - i1.5 - \text{pH}_0}{k_{\text{nion}}} + \frac{3}{i(k_{\text{ion}} - k_{\text{nion}})}\ln\left(\frac{k_{\text{ion}}}{k_{\text{nion}}}\right)\right) \tag{14}$$

with adjusted retention time

$$t'_{\text{R}} = t_{\text{d}} + t_{\text{s}} + \frac{\text{p}K_{\text{a,chrom}} + i1.5 - \text{pH}_0}{\dfrac{k_{\text{nion}}}{k_{\text{nion}}t_{\text{o}} - t_{\text{d}} - t_{\text{s}}}\left(\dfrac{\text{p}K_{\text{a,chrom}} - i1.5 - \text{pH}_0}{k_{\text{nion}}} + \dfrac{3}{i(k_{\text{ion}} - k_{\text{nion}})}\ln\left(\dfrac{k_{\text{ion}}}{k_{\text{nion}}}\right)\right)} \tag{15}$$

In the case of a pH gradient that starts at $\text{pH}_0 = \text{p}K_{\text{a,chrom}} - i1.5$ and ends at $\text{pH}_{\text{f}} = \text{p}K_{\text{a,chrom}} + i1.5$, eqs 14 and 15 simplify to

$$a_{\text{opt}} = \frac{3k_{\text{nion}}(\ln k_{\text{ion}} - \ln k_{\text{nion}})}{i(k_{\text{ion}} - k_{\text{nion}})(k_{\text{nion}}t_{\text{o}} - t_{\text{d}} - t_{\text{s}})} \tag{16}$$

$$t'_{\text{R}} = t_{\text{d}} + t_{\text{s}} + t_{\text{g,opt}} \tag{17}$$

where $t_{\text{g,opt}}$ is the optimal value of pH-gradient duration:

$$t_{\text{g,opt}} = \frac{a_{\text{opt}}}{\text{pH}_{\text{f}} - \text{pH}_0} = \frac{(k_{\text{ion}} - k_{\text{nion}})(k_{\text{nion}}t_{\text{o}} - t_{\text{d}} - t_{\text{s}})}{k_{\text{nion}}(\ln k_{\text{ion}} - \ln k_{\text{nion}})} \tag{18}$$

Equations 14−18 are valid only when the pH gradient starts at the column inlet before the adjusted retention time of the nonionized form of the analyte ($t_{\text{d}} + t_{\text{s}} < k_{\text{nion}}t_{\text{o}}$). Moreover, when the sum of dwell time and the pH-gradient starting time (t_{s}) approaches the adjusted retention time of the nonionized form of the analyte, $t_{\text{d}} + t_{\text{s}} \rightarrow k_{\text{nion}}t_{\text{o}}$, then the adjusted retention time attains $t'_{\text{R}} \rightarrow t_{\text{d}} + t_{\text{s}}$ and the optimal gradient steepness goes to infinity $a_{\text{opt}} \rightarrow \infty$. Since the steeper is the pH gradient the higher is the peak compression, one can anticipate that the best conditions to conduct the pH-gradient RP HPLC would be for a steep pH gradient developed just before the analyte elution from the column.

The convenient way to test analyte compression is by direct comparison of peak width that provided by the pH-gradient method yielding the same retention time. In such a situation the peak compression factor is given by

$$P_{\text{compr}} = \frac{W_{\text{pHgra}}(t_{\text{R}})}{W_{\text{iso}}(t_{\text{R}})} \tag{19}$$

where W_{pHgra} and W_{iso} are the peak width obtained at a pH gradient and isocratic conditions yielding the same retention time. When there is no pH-gradient effect on peak width this ratio should be 1. Otherwise it is lower, suggesting a peak compression. The isocratic peak widths were described by means of the following equation:

$$W_{\text{iso}} = 4\sqrt{\frac{t_{\text{R}}^2}{L/\text{HETP}} + \sigma_0^2} \tag{20}$$

where HETP is the isocratic local height equivalent to a theoretical plate of the column, L denotes column length, and σ_0^2 is an extracolumn band spreading. We observed that it was dependent on the organic modifier content and the mobile phase pH. The following equation was proposed to relate HETP and the mobile phase composition, φ and pH:

$$\mathrm{HETP}(\varphi, \mathrm{pH}) = h_1 + \frac{h_2}{1 + k_i(\varphi, \mathrm{pH})} \qquad (21)$$

MATERIALS AND METHODS

Experiments were done using a Merck-Hitachi LaChrome (Darmstadt, Germany; San Jose, CA) apparatus equipped with a diode array detector, autosampler, and thermostat. Chromatographic data were collected using a D-7000 HPLC system manager, version 3.1 (Merck-Hitachi). An XTerra MS C-18 column, 150 mm × 4.6 mm, particle size 5 μm (Waters Corporation, Milford, MA) was used. The system dwell volume V_d equaled 1.74 mL. A solution of 1% thiourea was injected to determine the column holdup volume, V_o. It has been found to be independent of the mobile phase composition and equaled 1.44 ± 0.02 mL. The extracolumn volume equaled 0.59 mL. It served to find extracolumn time that has been subtracted from all the retention times prior to any calculation. The extracolumn band spreading equaled $\sigma_0^2 = 0.022$ min^2. It was determined from the thiourea peak width measured without a column connected to the system, according to $\sigma_0^2 = (W/4)^2$. Chromatographic measurements were done at 25 °C with an eluent flow rate of 1.0 mL/min. All the reagents and the analytes employed were of a highest commercially available quality. Ketoprofen and papaverine were detected at their maximal absorbance (260 nm for ketoprofen, and 239 and 250 for the nonionized and the ionized forms, respectively, of papaverine). All the reagents and the analytes employed were of a highest commercially available quality. A peak width at the base has been calculated from the peak area and height according to $W = 2$(area/height). Prior to each measurement the column was equilibrated by passing six column volumes of the mobile phase.

The measurement and control of eluent pH is of great importance during the pH gradient. The details of proper assessment of pH in mixed organic/water mobile phases are discussed by Rosés.[15] In this work the pH of the mobile phase was measured after mixing the aqueous buffer and the organic modifier. The electrode system was calibrated with the usual aqueous standards. This led to the absolute pH scale, $^{s}_{w}$pH. This scale has been defined and recommended by IUPAC.[16] For simplicity, here the left side notation in pH and pK_a symbols has been omitted. The pH of the buffers was measured at 25 °C. The measurements were done with an HI 9017 pH meter (Hanna Instruments, Bedfordshire, U.K.).

We used a universal buffer to control pH changes of the mobile phase. The base buffer solution was formed using three compounds, each at a concentration of 0.008 M: citric acid (CIT), tris(hydroxymethyl)-aminomethane (Tris), and glycine (GLY). Buffer of pH = 10.50 (buffer D) was made by adding 3 M KOH to the base solution. Buffer of pH = 2.50 (buffer C) was made by adding the necessary amount of 1 M HCl. The mobile phases contained buffers D and C in different proportions and methanol as the organic modifier (solvent B). Prior to any experiments, the pH value has been determined for a multiple combination of B, C, and D, as presented in Figure 2. The linear interpolation served to find the pH value for the desired combination of mobile phase components. For the pH-gradient development the linear change of pH has been assumed (Figure 2) with initial and final pH (pH_0 and pH_f) determined by the extreme values.

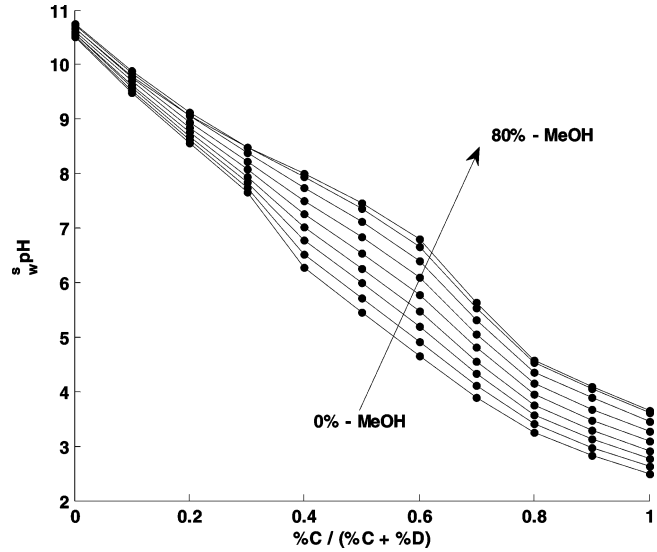

Figure 2. Experimental and linearly interpolated values of $^{s}_{w}$pH for different compositions of methanol (0, 10%, ..., 80%), buffer C of $^{w}_{w}$pH = 2.50, and buffer D of $^{w}_{w}$pH = 10.50. At constant organic modifier content the changes obtained by mixing buffer C and D were assumed to be linear.

Numerical analysis and data processing were done with Matlab software version 7.0 (The MathWorks, Inc., Natick, MA). The integration of eq 5 was done by means of the trapezoidal method implemented in the cumtrapz function. The nonlinear data-fitting problem was solved by the maximum likelihood approach. The minimum was found by means of a simplex algorithm implemented in fminsearch function.

RESULTS AND DISCUSSION

The validity of theory presented in this work was tested using two typical analytes: ketoprofen (weak acid) and papaverine (weak base). In order to apply the proposed theory, the parameters describing analyte retention need to be known. In our approach those parameters are the retention factors of the ionized and nonionized form of analyte (k_{w1} and k_{w2}) along with the S_1 and S_2 values describing dependence of the retention factor on organic modifier content. Additionally, the value of the dissociation constant p$K_{a,chrom}$ was used. Those parameters were estimated by fittings eq 3 to the isocratic experimental data. The peak width data served to find the relationship between HETP and both the pH and φ. Two parameters h_1 and h_2 were estimated by fitting eq 20 to the experimental peak widths. The data, along with model predictions, are presented in Figure 3.

The predicted retention times and peak widths are close to the experimental data. There are only small deviations for ketoprofen in the region of high pH, where the retention of the

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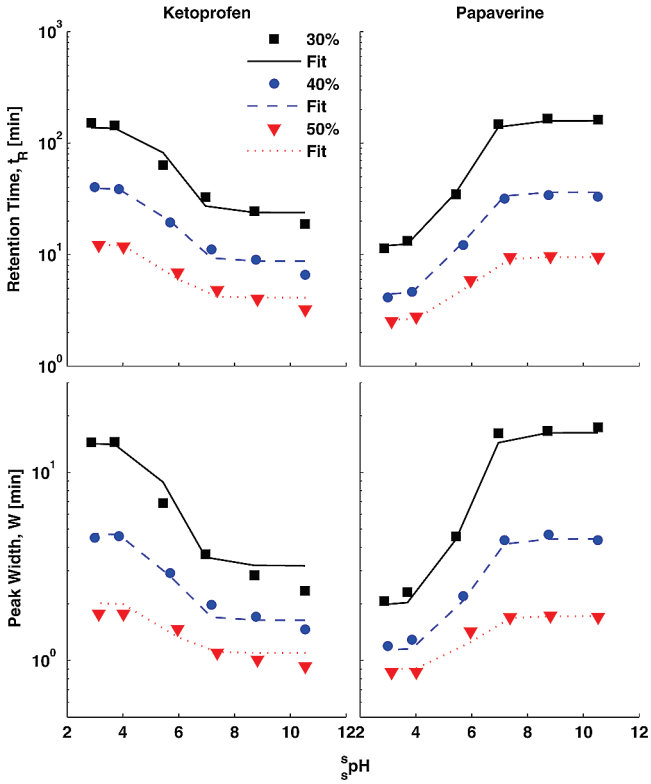

Figure 3. Experimental and model-predicted retention times and peak widths for ketoprofen (acid) and papaverine (base). The model predictions were obtained from eqs 3 and 20.

Table 1. Parameter Estimates Obtained by a Nonlinear Curve Fitting of Eq 3 to the Experimental Retention Times and Eq 20 to the Experimental Peak Widths Presented in Figure 3

	ketoprofen		papaverine	
retention time parameter	estimate	%CV	estimate	%CV
log $k_{w,1}$	3.66	3.21	2.69	3.53
log $k_{w,2}$	2.71	5.02	4.02	0.983
S_1	5.60	5.28	6.17	4.48
S_2	5.10	7.09	6.60	1.50
$pK_{a,chrom}$	5.45	2.24	6.14	0.531

peak width parameters	estimate	%CV
h_1	0.00865	8.4
h_2	0.136	8.0

analyte decreases contrary to the constant model predictions. We hypothesized that this is a consequence of the ion pair formation between the negatively charged form of ketoprofen and the positively charged form of the Tris buffer component. However, more work needs to be done to fully understand this unusual behavior.

The obtained parameter estimates are presented in Table 1. All the parameters were determined with a high precision, which is confirmed by the small coefficients of variation (%CV). The $pK_{a,chrom}$ differs from the literature aqueous pK_a since it corresponds to a certain methanol content (about 40%).[9]

In order to determine parameters of interest, another approach could be proposed, i.e., a series of organic modifier gradients conducted at different pHs and for different gradient durations.[12]

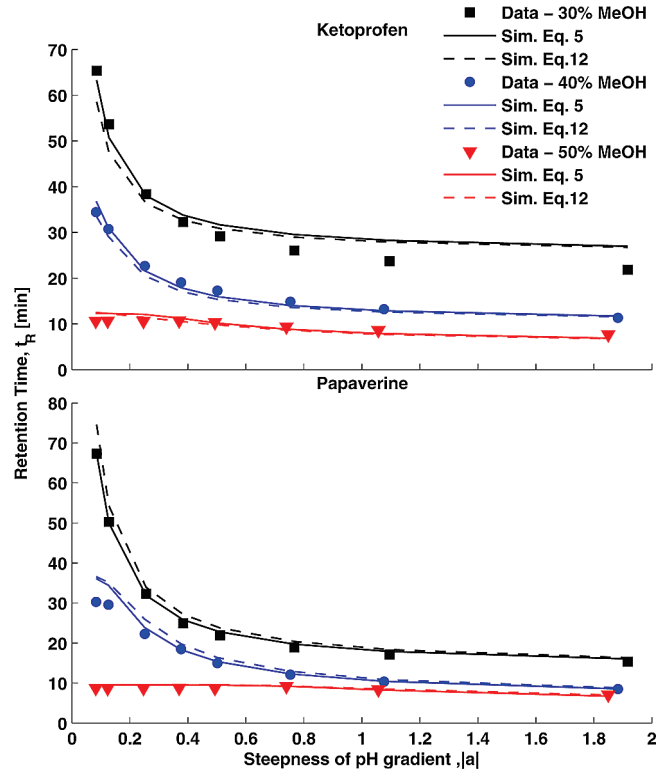

Figure 4. Experimental data and the simulated by means of eq 5 (—) and eq 12 (– – –) retention times during a series of pH gradients. The experiments were conducted at different MeOH contents in the eluent and for different gradient steepnesses *a*. The parameter estimates from Table 1 were used to perform simulations.

Certainly, if more detailed information about analyte retention is known the more complex function relation k and mobile phase composition can be considered, i.e., a quadratic relationship between log k and φ and/or a linear dependence of pK_a on φ.

Having the parameters of interest determined, it is feasible to predict the retention in any chromatographic mode with respect to any change in pH and/or methanol content in the eluent. To test the validity of our approach a series of pH-gradient experiments has been conducted for various pH-gradient steepnesses and for different methanol contents to assess if it is possible to predict pH-gradient retention by mean of eq 5 and to check if the proposed approximation (eq 12) holds. The results are presented in Figure 4. Both the full and the approximated equations yield data close to the experimental ones, suggesting that both models can be used to predict pH-gradient retention times. Also in general, the proposed approximation does not affect the predictions. The small deviations are a consequence of the model simplification and the assumption of the linear pH changes during the gradient, which in fact slightly deviate from the straight lines (Figure 2).

The peak compression phenomenon can be expected to occur in the pH-gradient mode. It results from different migration rates of analyte within the peak band. Because for some part of the pH gradient, the retention factor of the analyte at the beginning of the peak band is smaller (hence the analyte moves faster) than at the end of the peak. Figure 5 presents the changes of peak width for different pH-gradient steepnesses and for different methanol contents. In all cases the peak width decreases with the increase in the absolute value of a to some approximately constant value. For the very fast gradients (high $|a|$), the effect of

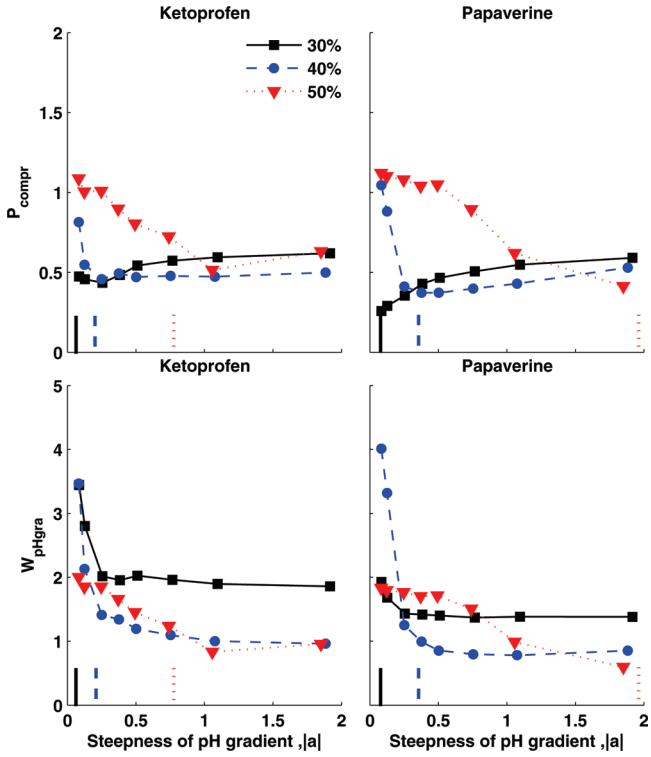

Figure 5. Experimental peak width, $W_{pHgra,}$ and peak compression factors, P_{compr}, calculated by means of eq 19 for a series of pH gradients with different gradient steepnesses. Vertical lines represent the optimal value of a (leading to the maximal peak compression) as calculated by means of eq 14. The methanol content is indicated in the first subplot.

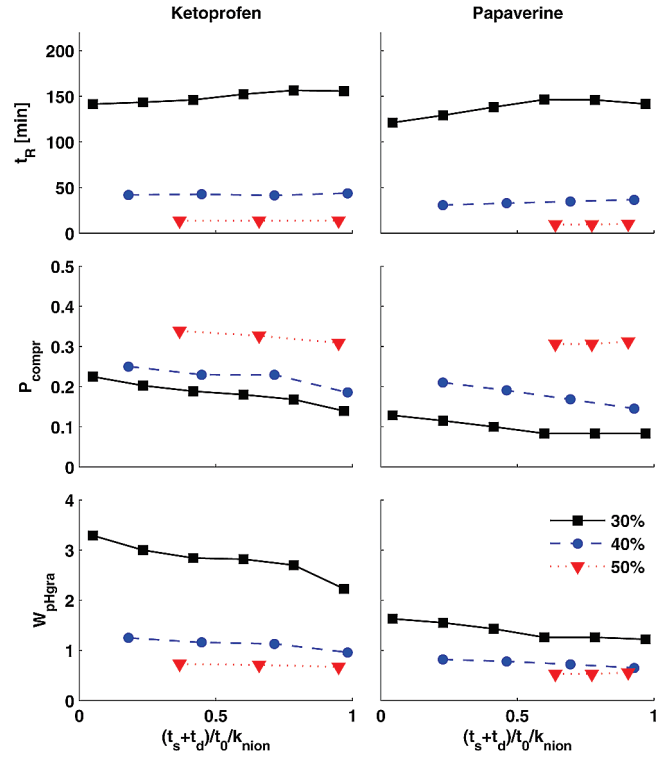

Figure 6. Experimental retention times, peak widths, W_{pHgra}, and peak compression factors, P_{compr}, calculated by means of eq 19 for a series of pH gradients with different starting times, t_s, and for optimal gradient steepness calculated by means of eq 14. The methanol content is indicated in the last subplot.

pH on retention is small since the pH changes affect analyte retention for a very short period of time; thus, most of the time the analytes migrate as ionized forms (the apparent isocratic conditions). Contrary, for small $|a|$, the peak width changes substantially and is mostly driven by changes in retention time.

It is very difficult to distinguish the contributions of pH gradient and band dispersion due to the time spent in the column on the peak width. In order to differentiate those two mechanisms, the peak compression factor has been proposed, as the ratio of peak width in a given pH gradient to the peak width that would be obtained in an isocratic mode at the conditions leading to the same retention time. It enabled direct determination of the pH-gradient parameters that would lead to the highest peak compression. The experimental results are presented in Figure 5, along with the theoretically predicted optimal values of gradient steepness, according to eq 14.

The relationship between peak compression factor (P_{compr}) and a elicits two distinctive phases. In the case of papaverine only one phase is present for 30% and 50% methanol due to a narrow range of tested a. In general, the P_{compr} initially decreases from 1 to the minimum and finally increases, however, to values lower than 1, suggesting that application of a less steep pH gradient than the optimal one also gives some peak compression. The minimal P_{compr} is very well predicted by the proposed equation (eq 14). The highest compression was found at low organic modifier contents. The minimum for papaverine and ketoprofen equaled 0.2 and 0.4, respectively, for 30% methanol in the mobile phase.

From both the theoretical and practical points of view one can also obtain a high peak compression by applying a short pH gradient just before the analyte elution for an otherwise isocratic separation. To illustrate that, we have performed a series of pH gradients with different pH-gradient starting times (t_s) and with optimal gradient steepness (a_{opt}), calculated by means of eq 14. The results for ketoprofen and papaverine are presented in Figure 6. The peak widths for both ketoprofen and papaverine were higher for the low methanol contents. It is a consequence of higher retention times. Contrarily, the peak compression factor was higher for low methanol contents, suggesting that for the larger retention time one can more easily obtain narrow peaks, however, at the cost of the longer separation. As was expected, the peak width and peak compression factor decreased with increasing t_s as a result of increase in gradient steepness a_{opt} (eq 14) and was smallest when $t_s + t_d$ approached the adjusted retention factor of the nonionized form of analyte ($t_o k_{nion}$). Irrespective of the value of t_s, the P_{comp} was in the range of 0.2−0.3, suggesting that 3−5 times higher peaks were obtained. Figure 7 illustrates the benefits of pH gradient in the selected separations along with the isocratic separation of the nonionized form of the analyte. It is clear that the application of the pH gradient has led to the improved shape of peaks, decreased peak width, and increased peak height.

The theoretically and experimentally proposed mode of pH-gradient RP HPLC in this work may be applied to analytes with more than one dissociation group. One needs to consider only a narrow range of pH around one of the dissociation steps. However, it is much more difficult to apply the linear pH gradient to optimize the separation of a multicomponent mixture in order to obtain

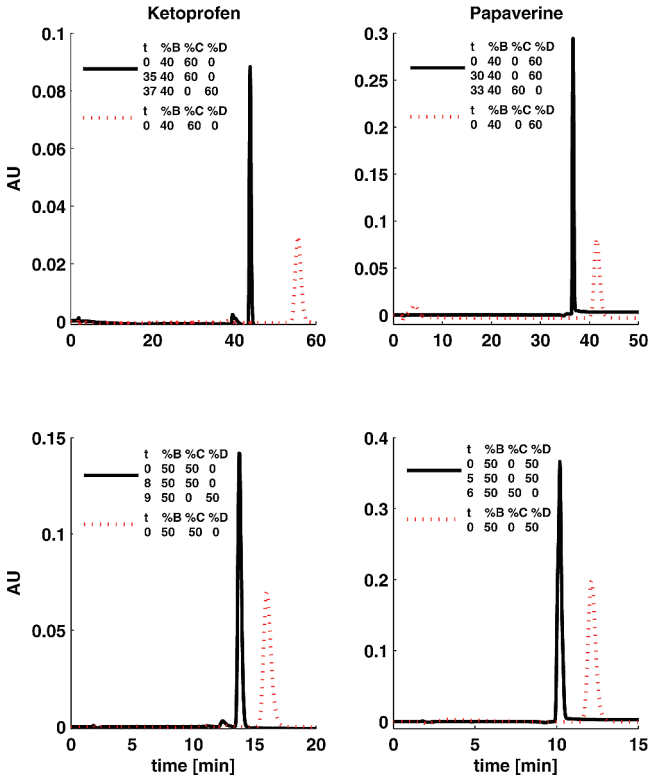

Figure 7. Experimental chromatograms obtained for optimized pH-gradient conditions and isocratic conditions at the pH ensuring the appearance of the completely nonionized form of the analyte. The applied pump programs are given within each subplot.

the desired resolution and peak compression for all the analytes. Since there is only a narrow range of pH-gradient parameters ensuring maximal peak compression, it is difficult to find the optimal conditions for a set of analytes, especially with different retention behaviors. Certainly, that is possible for a limited number of analytes with similar retention (two or three), e.g., one compound along with an internal standard in the quantitative analysis just following the above proposed theory.

CONCLUSION

The pH gradient is a very attractive method of analyzing ionizable analytes. The best conditions for conducting the pH gradient can be relatively easily found by means of the proposed theory. It requires a series of preliminary experiments to determine the parameters describing analyte retention. On the basis of them, one can use the proposed method to find the best pH gradient for a given purpose. We have shown the evident benefits of this technique, as it may lead to a substantial peak compression (up to 0.2) and completely removes peak tailing. In our opinion any isocratic separation can be transferred to the pH-gradient mode by appropriately selecting the pH-gradient program. It is also applicable to the analyte quantification, since a peak's sensitivity and limit of quantification can be significantly increased.

ACKNOWLEDGMENT

The project was financially supported by a Grant from the Ministry of Science and Higher Education, Warsaw, Poland (Grant N N405 376037), by the START fellowship from the Foundation for Polish Science, and by a Grant from Iceland, Liechtenstein, and Norway through the EEA Financial Mechanism via Homing Program from the Foundation for Polish Science.

Received for review January 5, 2010. Accepted March 13, 2010.

AC100023Z