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pH gradient high-performance liquid chromatography: theory and applications

Roman Kaliszan*, Paweł Wiczling, Michał J. Markuszewski

Department of Biopharmaceutics and Pharmacodynamics, Medical University of Gdańsk, Gen. J. Hallera 107, Gdańsk 80-416, Poland

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Abstract

pH gradient high-performance liquid chromatography (HPLC) is a method of reversed-phase high-performance liquid chromatography suitable for ionogenic substances. It consists in programmed increase during the chromatographic process of the eluting strength of eluent with respect to the analytes separated. On the analogy of the conventional organic modifier gradient reversed-phase HPLC, in the pH gradient approach the eluting strength of the mobile phase increases due to its changing pH: increasing in case of acids or decreasing in case of bases. At the same time the content of organic modifier remains constant. A theory of the pH gradient HPLC has been elaborated. The resulting mathematical model is easily manageable. Its ability to predict changes in retention and separation of analytes following the changes in chromatographic conditions is demonstrated. The pH gradient method is uniquely suitable to determine pK_a values of analytes. An equation is presented allowing to calculate pK_a values basing on appropriate retention data. The effects on pK_a are discussed of the concentration of methanol in the mobile phase. The RP HPLC-derived pK_a data correlate to the reference pK_a values ($^{w}_{w}$pK_a) but are not identical. That may be explained by the effects on the chromatographically determined pK_a of the specific interactions of analytes with stationary phases. The proposed pH gradient RP HPLC procedure offers a fast and convenient means to get comparable acidity parameters for larger series of compounds, like drug candidates, also when the analytes are available only in minute amounts and/or as complex mixtures.

Keywords: pH gradient; Mathematical modeling

1. Introduction

Retention of ionogenic analytes in reversed-phase HPLC is known to strongly depend on pH of the eluent. That offers convenient means to rationally modify separation of ionizable compounds. A theory for isocratic systems with a buffered water mobile phase was given by Horvath et al. [1]. Van de Venne et al. [2] extended the studies on the relationships between eluent pH and analyte retention to the methanol-water mobile phases. Appropriate mathematical formalistics was reported by Lopez Marques and Schoenmakers [3]. Rules of selection of pH of the mobile phase to optimize separations have been defined by Snyder and coworkers [4]. The effects of organic modifiers on acid–base equilibria in mobile phases, along with a critical review of a vast literature on the subject, have been defined by Roses and Bosch [5,6]. An up-to-date presentation of the subject is due to Barbosa and coworkers [7]. The effect of pH on retention of bases was also examined by LoBrutto et al. [8].

A narrow range pH gradient was employed by Little et al. [9] to improve separation of some acidic analytes. A systematic analytical use of approach using a wide-range the pH gradient became feasible after application of universal buffers [10,11]. The empirical approach was supported with an approximate theory [12,13]. Recently we succeeded in providing a comprehensive theoretical background for the method [14,15]. The pH gradient RP HPLC is executed by linearly increasing (in case of acid analytes) or decreasing (in case of bases) the pH of the eluent of a constant organic solvent content, thus providing a functional increase in analyte dissociation and consequently, a decrease in its retention. A stringent theory allows predictions of gradient retention at changing chromatographic conditions.

Acidity parameter pK_a is of utmost importance to predict physicochemical, material and biological properties of individual members of a congeneric series of compounds. Specifically, pharmacokinetics (ADME–absorption, distribution, metabolism, excretion) of xenobiotics depends

* Corresponding author. Tel.: +48 583493260; fax: +48 583493262.
E-mail address: roman.kaliszan@amg.gda.pl (R. Kaliszan).

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on their pK_a. Mostly, because pK_a affects apparent drug lipophilicity. Therefore, modern approaches to the search for new drugs, like high-throughput screening (HTS), require ready access to the pK_a data of drug candidates, often supplied as complex mixtures, e.g., by combinatorial chemistry [16].

pH-metric titrations and spectrophotometric analysis are routinely used for pK_a determination [17]. However there are limitations of those procedures, like poor compound solubility (pH-metry) or a lack of a chromophore, so that the ionized and the non-ionized molecules cannot be differentiated spectrophotometrically [18]. Because many compounds of pharmaceutical interest tend to be poorly soluble in water and are usually not readily available in a high purity form, the classical techniques are not practical in HTS.

Liquid chromatography (LC) may be applied to determine dissociation constants of ionogenic substances. The advantages of the LC methods are: a very small quantity of the analyte required for assay and no requirement of high purity of the sample. Among the LC techniques used for determinations of pK_a are reversed-phase TLC and HPLC [19], ion chromatography [20] and ion-exchange chromatography [21]. Capillary electrophoresis has also since some time been used for that purpose [22,23].

Effects of pH on the mobile phase on isocratic RP HPLC retention factor, k, has been the subject of numerous publications [1,3,24–26]. On the basis of that relationship, there is a means to determine pK_a from a series of 8–10 isocratic retention measurements at different pH of the eluent. Isocratic (actually polycratic) HPLC determinations of pK_a are time consuming, however, and nowadays medicinal chemistry needs a faster method.

In liquid chromatography with gradient elution the fundamental equation describing analyte retention, valid for either the organic modifier gradient or the pH gradient, is:

$$\int_0^{V'_R} \frac{1}{V_0}\frac{dV}{k_i} = 1 \tag{1}$$

where V is the cumulative volume of the mobile phase flowing through the column since the beginning of gradient, V_0 the column void ("dead") volume and k_i the analyte retention factor, corresponding to the composition of the mobile phase at column inlet. The passage of a differential volume element, dV, of the mobile phase through the column results in a fractional band migration $dx = dV/(V_0 k_i)$. When the total volume of mobile phase introduced onto the column equals the reduced retention volume, $V'_R = V_R - V_0$, the sum of fractional migration $\sum dx = 1$. Analogously:

$$\int_0^{t'_R} \frac{1}{t_0}\frac{dt}{k_i} = 1 \tag{2}$$

where $t'_R = t_R - t_0$ denotes the reduced retention time [27].

Description of retention factor of ionizable compounds as a function of pH at constant organic modifier content in the mobile phase has been established. The rationale is as follows [14]. For monoprotic acids the retention factor, k, is:

$$k = f_{[HA]}k_{[HA]} + f_{[A^-]}k_{[A^-]} \tag{3a}$$

And for monoprotic bases it is:

$$k = f_{[BH^+]}k_{[BH^+]} + f_{[B]}k_{[B]} \tag{3b}$$

where subscripts identify specific dissociated and non-dissociated forms of acid or base and f denotes mole fraction of individual forms. There are known the following relationships:

$$f_{[HA]} = \frac{1}{K_a/[H^+] + 1} \tag{4}$$

$$f_{[A^-]} = 1 - f_{[HA]} \tag{5}$$

with K_a denoting analyte dissociation constant and $[H^+]$ symbolizing hydrogen ion concentration. Eqs. (4) and (5) are for acids. Analogous equations hold for bases. Now, Eq. (3) can be transformed to the following well-established relationships.

For acids:

$$k = \frac{k_{[HA]} + k_{[A^-]}10^{pH-pK_a}}{1 + 10^{pH-pK_a}} = \frac{k_{[A^-]} + k_{[HA]}10^{pK_a-pH}}{1 + 10^{pK_a-pH}} \tag{6a}$$

For bases:

$$k = \frac{k_{[BH^+]} + k_{[B]}10^{pH-pK_a}}{1 + 10^{pH-pK_a}} = \frac{k_{[B]} + k_{[BH^+]}10^{pK_a-pH}}{1 + 10^{pK_a-pH}} \tag{6b}$$

In the pH gradient RP HPLC procedure the pH of the eluent changes linearly with time:

$$pH = pH_0 + at \tag{7}$$

where pH_0 is the starting pH and a is the programmed rate of pH change. Therefore, for retention factor, k_i, at $pH = pH_0 + at$, Eqs. (6a) and (6b) can be rewritten in the forms of Eqs. (8a) and (8b), for acids and bases, respectively:

$$k_i = \frac{k_{[A^-]} + k_{[HA]}10^{pK_a-(pH_0+at)}}{1 + 10^{pK_a-(pH_0+at)}} \tag{8a}$$

$$k_i = \frac{k_{[BH^+]} + k_{[B]}10^{(pH_0+at)-pK_a}}{1 + 10^{(pH_0+at)-pK_a}} \tag{8b}$$

Substituting Eqs. (8a) and (8b) into Eq. (2) gives the following equations for pH gradient HPLC.

For acids:

$$\int_0^{t'_R} \frac{1}{t_0}\frac{1 + 10^{pK_a-(pH_0+at)}}{k_{[A^-]} + k_{[HA]}10^{pK_a-(pH_0+at)}}dt = 1 \tag{9a}$$

For bases:

$$\int_0^{t'_R} \frac{1}{t_0}\frac{1 + 10^{(pH_0+at)-pK_a}}{k_{[BH^+]} + k_{[B]}10^{(pH_0+at)-pK_a}}dt = 1 \tag{9b}$$

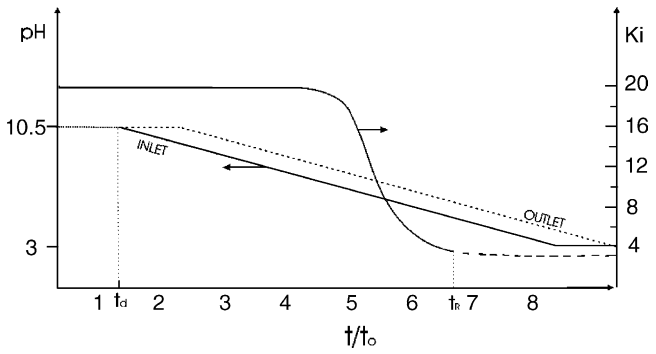

Fig. 1. Changes of instantaneous retention factor, k_i, during pH gradient elution for a hypothetical base of $pK_a = 6$.

Placing ($t' = k_{t_0}$) in Eqs. (9a) and (9b), one gets:

For acids:

$$\int_0^{t'_R} \frac{1 + 10^{pK_a - (pH_0 + at)}}{t'_{[A^-]} + t'_{[HA]} 10^{pK_a - (pH_0 + at)}} dt = 1 \quad (10a)$$

For bases:

$$\int_0^{t'_R} \frac{1 + 10^{(pH_0 + at) - pK_a}}{t'_{[BH^+]} + t'_{[B]} 10^{(pH_0 + at) - pK_a}} dt = 1 \quad (10b)$$

During the pH gradient elution, the actual pH of the mobile phase in the column at time t is delayed for a value of the HPLC system dwell time, t_d (Fig. 1). Therefore:

$$pH = pH_0 \quad \text{when} \quad 0 \le t \le t_d \quad (11a)$$

$$pH = pH_0 + a(t - t_d) \quad \text{when} \quad t_d \le t \le t_G + t_d \quad (11b)$$

$$pH = pH_0 + at_G \quad \text{when} \quad t \ge t_G + t_d \quad (11c)$$

where pH_0 denotes pH at the beginning of gradient run, t_G the time of gradient run and a the rate of pH change which is equal to $\Delta pH/t_G$.

Taking gradient delay, t_d, into account, one obtains the following equations describing retention in pH gradient RP HPLC.

For acids:

$$\int_0^{t'_R} \frac{1}{t'_{[HA]}} dt = 1 \quad \text{when} \quad t'_R \le t_d \quad (12a)$$

$$\int_0^{t_d} \frac{1}{t'_{[HA]}} dt + \int_{t_d}^{t'_R} \frac{1 + 10^{pK_a - (pH_0 + a(t - t_d))}}{t'_{[A^-]} + t'_{[HA]} 10^{pK_a - (pH_0 + a(t - t_d))}} dt = 1$$
$$\text{when} \quad t_d \le t'_R \le t_G + t_d \quad (12b)$$

$$\int_0^{t_d} \frac{1}{t'_{[HA]}} dt + \int_{t_d}^{t_g + t_d} \frac{1 + 10^{pK_a - (pH_0 + a(t - t_d))}}{t'_{[A^-]} + t'_{[HA]} 10^{pK_a - (pH_0 + a(t - t_d))}} dt + \int_{t_g + t_d}^{t'_R} \frac{1}{t'_{[A^-]}} dt = 1 \quad \text{when} \quad t'_R \ge t_G + t_d \quad (12c)$$

For bases:

$$\int_0^{t'_R} \frac{1}{t'_{[B]}} dt = 1 \quad \text{when} \quad t'_R \le t_d \quad (13a)$$

$$\int_0^{t_d} \frac{1}{t'_{[B]}} dt + \int_{t_d}^{t'_R} \frac{1 + 10^{(pH_0 + a(t - t_d)) - pK_a}}{t'_{[HB^+]} + t'_{[B]} 10^{(pH_0 + a(t - t_d)) - pK_a}} dt = 1$$
$$\text{when} \quad t_d \le t'_R \le t_G + t_d \quad (13b)$$

$$\int_0^{t_d} \frac{1}{t'_{[B]}} dt + \int_{t_d}^{t_g + t_d} \frac{1 + 10^{(pH_0 + a(t - t_d)) - pK_a}}{t'_{[HB^+]} + t'_{[B]} 10^{(pH_0 + a(t - t_d)) - pK_a}} dt + \int_{t_g + t_d}^{t'_R} \frac{1}{t'_{[BH^+]}} dt = 1 \quad \text{when} \quad t'_R \ge t_G + t_d \quad (13c)$$

Eqs. (12a) and (13a) describe analyte retention at constant pH (isocratic conditions with regards to eluent pH), when $t'_{[HA]}$ and $t'_{[B]}$ do not change with time and apply to the case when the analytes are washed out from the column prior to the start of pH gradient. Thus, Eqs. (12a) and (13a) account for isocratic part of the chromatographic run and are relevant in the case of substances of a low k and a high (bases) or a low (acids) pK_a, when the applied gradient of pH comprises the pH values far from the analyte's pK_a. Such a situation is of no usual RP HPLC separation concern.

Eqs. (12b) and (13b) describe the case when the analyte retention time is between the start and the end of pH gradient. These equations are sums of two integrals. First integral refers to isocratic retention, as described by Eqs. (12a) and (13a), respectively. Second integral accounts for the retention at actual pH gradient conditions. Actually, when values of $t'_{[HA]}$ and $t'_{[B]}$ are relatively large (as often happens in practice), the first part in Eqs. (12b) and (13b) can be neglected and then the solution simplifies. Nonetheless, the solution of complete Eqs. (12b) and (13b) is also possible as we demonstrated in our previous work [14]. Thus, the solution for bases is:

$$\left[\left(\frac{t'_{[HB^+]}}{t'_{[B]}} - 1 \right) \log \frac{t'_{[HB^+]} + t'_{[B]} 10^{pH^{**} - pK_a}}{t'_{[HB^+]} + t'_{[B]} 10^{pH_0 - pK_a}} - pH_0 + pH^{**} \right] = \left(1 - \frac{t_d}{t'_{[B]}} \right) a t'_{[HB^+]} \quad (14a)$$

Analogously, the equation describing pH-gradient RP HPLC of acids has the form:

$$\left[\left(\frac{t'_{[A^-]}}{t'_{[HA]}} - 1 \right) \log \frac{t'_{[A^-]} + t'_{[HA]} 10^{pK_a - pH^{**}}}{t'_{[A^-]} + t'_{[HA]} 10^{pK_a - pH_0}} + pH_0 - pH^{**} \right] = - \left(1 - \frac{t_d}{t'_{[HA]}} \right) a t'_{[A^-]} \quad (14b)$$

The symbol pH^{**} denotes pH at the end of the column at the moment when the analyte leaves it, i.e., $pH^{**} = pH_0 + a(t'_R - t_d)$.

The third situation in pH gradient RP HPLC, accounted for by Eqs. (12c) and (13c), happens when the analyte's reduced retention time, t'_R, is longer than the sum of the gradient time, t_G, and the gradient delay time, t_d. The meaning of the first two terms in Eqs. (12c) and (13c) remains as explained above. The third interval describes analyte elution after completing the gradient program, i.e., at isocratic pH conditions formed after attaining the final pH denoted here as pH_F, which maintains a full ionization of the analyte. Solution of Eqs. (12c) and (13c) is like that given above for Eqs. (12b) and (13b) with one difference, namely the $pH_0 + a(t - t_d)$ for the time of chromatographic run $t \geq t_G + t_d$ equals the pH at the end of gradient program, pH_F. In effect, the general equation is for bases:

$$\left[\left(\frac{t'_{[HB^+]}}{t'_{[B]}} - 1\right)\log\frac{t'_{[HB^+]} + t'_{[B]}10^{pH_F - pK_a}}{t'_{[HB^+]} + t'_{[B]}10^{pH_0 - pK_a}} - pH_0 + pH_F\right] = \left(1 - \frac{t_d}{t'_{[B]}} - \frac{t'_R - t_G - t_d}{t'_{[HB^+]}}\right)at'_{[HB^+]} \quad (15a)$$

And for acids:

$$\left[\left(\frac{t'_{[A^-]}}{t'_{[HA]}} - 1\right)\log\frac{t'_{[A^-]} + t'_{[HA]}10^{pK_a - pH_F}}{t'_{[A^-]} + t'_{[HA]}10^{pK_a - pH_0}} + pH_0 - pH_F\right] = -\left(1 - \frac{t_d}{t'_{[HA]}} - \frac{t'_R - t_G - t_d}{t'_{[A^-]}}\right)at'_{[A^-]} \quad (15b)$$

Eqs. (14a), (14b), (15a) and (15b) can be rearranged to the respective forms, from which the pK_a parameter can be calculated. For bases, when $t_d \leq t'_R \leq t_G + t_d$:

$$pK_a = pH^{**} + \log\frac{t'_{[B]}}{t'_{[HB^+]}}\frac{10^{-at'_{[HB^+]}(t'_{[B]} - t'_R)/(t'_{[B]} - t'_{[HB^+]})} - 1}{1 - 10^{at'_{[B]}(t'_R - t'_{[HB^+]})/(t'_{[B]} - t'_{[HB^+]})^{-at_d}}} \quad (16a)$$

For acids, when $t_d \leq t'_R \leq t_G + t_d$

$$pK_a = pH^{**} - \log\frac{t'_{[HA]}}{t'_{[A^-]}}\frac{10^{at'_{[A^-]}(t'_{[HA]} - t'_R)/(t'_{[HA]} - t'_{[A^-]})} - 1}{1 - 10^{-at'_{[HA]}(t'_R - t'_{[A^-]})/(t'_{[HA]} - t'_{[A^-]}) - at_d}} \quad (16b)$$

For bases, when $t'_R \geq t_G + t_d$:

$$pK_a = pH_F + \log\frac{t'_{[B]}}{t'_{[HB^+]}}\frac{10^{at'_{[B]}(t'_R - t'_{[HB^+]})/(t'_{[B]} - t'_{[HB^+]}) - at_d - at_G} - 1}{1 - 10^{at'_{[B]}(t'_R - t'_{[HB^+]})/(t'_{[B]} - t'_{[HB^+]}) - at_d}} \quad (17a)$$

For acids, when $t'_R \geq t_G + t_d$

$$pK_a = pH_F - \log\frac{t'_{[HA]}}{t'_{[A^-]}}\frac{10^{-at'_{[HA]}(t'_R - t'_{[A^-]})/(t'_{[HA]} - t'_{[A^-]}) + at_d + at_G} - 1}{1 - 10^{-at'_{[HA]}(t'_R - t'_{[A^-]})/(t'_{[HA]} - t'_{[A^-]}) + at_d}} \quad (17b)$$

To determine pK_a, a proper measurement of eluent pH is a precondition. Various procedures of measurement of pH of the HPLC mobile phases and the specific pH and pK_a scales to which they lead, have recently been discussed in details for methanol/water eluent systems [5,7,28]. The most appropriate seems to be a procedure consisting in measurements the pH of the mobile phase after mixing the aqueous buffer and the organic modifier. The electrode system can be calibrated with the usual aqueous standards. This leads to the absolute pH scale, ${}^{s}_{w}pH$. When the electrode system is calibrated with the same mixed organic solvent like the mobile phase, the ${}^{s}_{s}pH$ scale is obtained. These scales have eventually been defined and recommended by IUPAC [29]. When the pK_a value is calculated based on the retention data obtained using a given mobile phase, pH of which has been expressed in one of these two scales, the thermodynamically meaningful dissociation constant of the compound is also in the same scale (${}^{s}_{w}pK_a$ or ${}^{s}_{s}pK_a$).

In this work the determinations of pH were carried out at the presence of aqueous standards, taking into consideration the influence of the organic modifier (methanol). When no notation precedes the pH and/or pK_a symbols then the respective values apply to the ${}^{s}_{w}pH$ and/or ${}^{s}_{w}pK_a$ scale.

Normally, to obtain measurable RP HPLC retention parameters an organic modifier must be added to the mobile phase, especially in case of water insoluble substances. However, in biological systems drugs are assumed to encounter a mainly aqueous environment. Therefore, methods to determine aqueous pK_a (${}^{w}_{w}pK_a$) are desired. To obtain ${}^{w}_{w}pK_a$ parameters from the chromatographically determined ${}^{s}_{w}pK_a$ or ${}^{s}_{s}pK_a$ values an extrapolation of data obtained at several mobile phase compositions or some semiempirical corrections have been suggested [30,31]. To obtain extrapolated ${}^{w}_{w}pK_a$ data, the Yasuda–Shedlovsky relationship is recommended of the following form [32,33]:

$${}^{s}_{s}pK_a + \log[H_2O] = \frac{a}{{}^{s}_{\varepsilon}} + b \quad (18)$$

The ${}^{w}_{w}pK_a$ is obtained from Eq. (18) by extrapolation to the inverse of 78.3 (the dielectric constant of water) when taking $\log[H_2O] = 55.5$ (the molar concentration of pure water). The procedure was applied to potentiometric titration data [34] as well as to pK_a data from isocratic HPLC [35,36].

The above derived equations describing pH gradient RP HPLC (Eqs. (14) and (15)) can be used for prediction of retention of individual analytes and hence, for rational optimization of separation conditions in both gradient and isocratic modes. Solution of Eqs. (14) and (15) is done

numerically, employing standard mathematical programs, like Excel. The pK_a parameters can be directly calculated from Eqs. (16) and (17).

To use appropriate equation one needs input data from two experiments, both at a constant pH providing complete suppression of analyte ionization but applying different organic-water gradients. From these experiments, an appropriate organic modifier concentration may be evaluated from the linear solvent strength (LSS) theory [37], allowing for a range of well measurable retention factors of the analyte under study. At the same time, one can calculate log k_w parameter corresponding to pure water eluent and considered to be the most reliable chromatographic measure of analyte lipophilicity [38]. Then, a programmed pH gradient run is performed with a constant concentration of organic modifier but at the pH range which provides a full complete suppression of ionization of the analyte at the beginning of the gradient and its total ionization at gradient end. One additional injection of the analyte after completing the pH gradient program provides the retention factor of its ionized form.

2. Experimental section

2.1. Equipment

The HPLC system applied was Merck-Hitachi LaChrome (Darmstadt, Germany-San Jose, CA, USA) of the dwell volume, V_d, of 1.4 ml, equipped with a diode array detector, autosampler and thermostat. Chromatographic data were collected using D-7000 HPLC System Manager, version 3.1 (Merck-Hitachi). The column was XTerra MS C-18, 150 mm × 4.6 mm i.d., particle size 5 μm (Waters Corporation, Milford, MA, USA), packed with octadecylbonded silica. Mobile phases contained methanol as the organic modifier. Water or buffers of fixed pH formed the aqueous component of the eluent. 1% urea was used as the column dead volume, V_0, marker. The dead volume such determined was 1.64 ± 0.02 ml. The injected sample volume was 10 μl. The analytes were dissolved in methanol at concentration of 0.5 mg/ml. Chromatographic measurements were done at 35 ± 0.1 °C with eluent flow-rate of 1.5 ml/min. All the reagents and analytes employed were of a highest commercially available quality.

2.2. Mobile phase used in experiments on prediction of pH gradient retention [14]

Universal buffer consisted of parts I and II. Basic solvent was formed by three acids, all at concentrations of 0.004 M: phosphoric, acetic and boric. Buffer I of ${}^{w}_{w}\mathrm{pH} = 3.00$ was made from that basic solvent by adjusting pH with 1 M HCl. Buffer II of ${}^{w}_{w}\mathrm{pH} = 10.50$ was prepared by pH adjustment with 1 M NaOH. The measurements were done with an HI 9017 pH-meter (Hanna Instruments, Bedfordshire, UK). The linear changes in pH of mobile phase were obtained by mixing of buffer I with buffer II. The values of pH used in calculations were obtained as described below.

2.3. Mobile phase used in experiments on pK_a determination [15]

The buffer described above was difficult to use because of its tendency to precipitate in spite of the applied increased temperature (35 °C) of the chromatographic process. We succeeded in obtaining another universal buffer devoid of that shortage. Again, mobile phase contained methanol as the organic modifier (solvent B). Buffers of ${}^{w}_{w}\mathrm{pH} = 3.00$ (buffer I) and ${}^{w}_{w}\mathrm{pH} = 10.50$ (buffer II), mixed at various proportions, formed the aqueous component of the eluent. Essential aqueous solvent formed three compounds, each at the concentration of 0.004 M: citric acid (CIT), tris-(hydroxymethyl)aminomethane (TRIS) and 3-(cyclohexyloamino)-1-propane sulfonic acid (CAPS). Buffer I was made from that solvent by adjusting pH with 1 M HCl. Buffer II was prepared by pH adjustment with 1 M NaOH. During the pH gradient run buffers II, together with a fixed content of methanol, and I were mixed in a mix chamber. The composition of the buffer was found in preliminary experiments to provide the linear change of pH during the pH gradient runs at various methanol content (Fig. 2). Because it would be difficult to measure pH during the gradient run, the changes of ${}^{s}_{w}\mathrm{pH}$ with the content of methanol were determined for solutions of buffer I and buffer II (Fig. 3). The actual ${}^{s}_{w}\mathrm{pH}$ during the pH gradient run was calculated by Eq. (19) (assuming linear changes of pH) employing necessary data obtained by the regression equations given in Fig. 3:

$$ {}^{s}_{w}\mathrm{pH} = {}^{s}_{w}\mathrm{pH}_0 + \frac{{}^{s}_{w}\mathrm{pH}_F - {}^{s}_{w}\mathrm{pH}_0}{t_G} t = {}^{s}_{w}\mathrm{pH}_0 + at \qquad (19) $$

In Eq. (19) ${}^{s}_{w}\mathrm{pH}_0$ is the value at the beginning of the gradient run, ${}^{s}_{w}\mathrm{pH}_F$ is the value at the end of the gradient (both for a given constant content of methanol), t_G is time

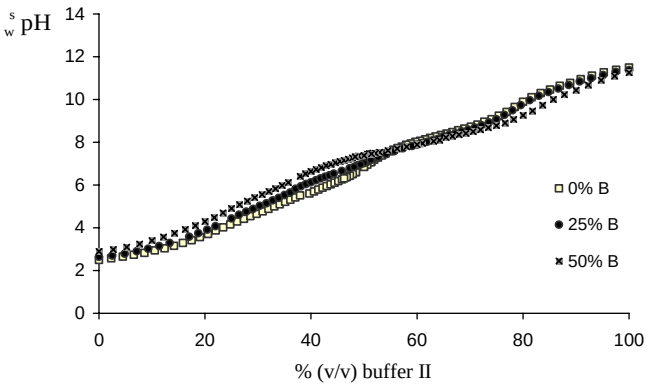

Fig. 2. ${}^{s}_{w}\mathrm{pH}$ of the solution formed by mixing of buffer I and II in the presence of different methanol contents.

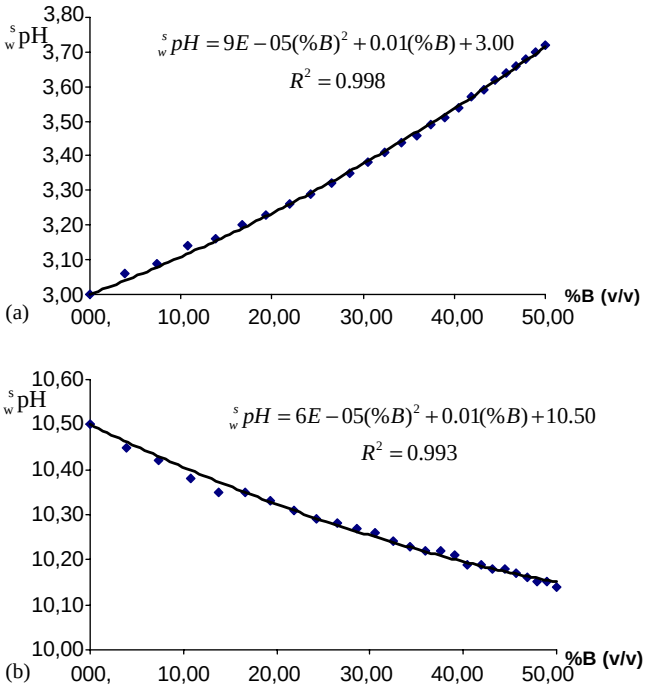

Fig. 3. Changes in $^{s}_{w}$pH with increasing content of methanol (% B) in solution: buffer of $^{w}_{w}$pH 3.00 (a); buffer of $^{w}_{w}$pH 10.50 (b). *R* is correlation coefficient.

of the gradient run and *a* is the programmed steepness of the pH gradient.

3. Results and discussion

Experiments on prediction of retention depending on pH gradient conditions began with a series of measurements for a set of five basic test analytes. In Fig. 4 exemplary pH gradient chromatograms of a test mixture of analytes are compared as determined experimentally and calculated from our model. It can be noted that pH gradient, provides relatively narrow, symmetrical peaks of approximately the same width, with minimized tailing. That reduced peak tailing is of special value in case of basic analytes for which the tailing is a serious problem with the standard HPLC procedures. The decreased peak tailing might be due to peak compression [39]. That may be explained as follows. The pH of eluent is linearly decreasing over the time during the pH gradient run in case of bases. At any site in the column, the analyte molecules passing through it are exposed to a weaker eluent than the molecules which pass through it later. A stronger eluent (lower pH) pushes bases faster than a weaker

Table 1
Experimental data used for calculation of $^{s}_{w}pK_a$ values of test analytes chromatographed on XTerra MS C-18 column

Analyte	$t_{[B]}$	$t_{[HB^+]}$	t_R	$^{s}_{w}pK_a$ (HPLC)	$^{w}_{w}pK_a$ (lit)
Aniline	8.67	1.76	8.16	4.87	4.63
p-Anisidine	11.41	1.84	8.59	5.29	5.34
2-Amino-5-nitropyridine	16.37	1.65	7.52	6.83	7.22
2-Methylobenzimidazole	23.09	2.96	8.93	6.32	6.19
Morphine	42.08	2.05	7.79	7.43	8.21

$t_{[B]}$ and $t_{[HB^+]}$ are isocratic retention times (in min) of non-dissociated and dissociated forms, respectively; t_R is the pH-gradient retention time determined with pH gradient from 10.50 to 3.00 formed within gradient time, t_G, of 8 min. Retention data were obtained on XTerra MS C-18 column with mobile phase containing 7% (v/v) of methanol flowing with the rate of 1.5 ml/min at 35 °C. Values of $^{s}_{w}pK_a$ (HPLC) calculated for these conditions and the $^{w}_{w}pK_a$ data taken from literature [40–42] were used for retention predictions at all the other conditions applied.

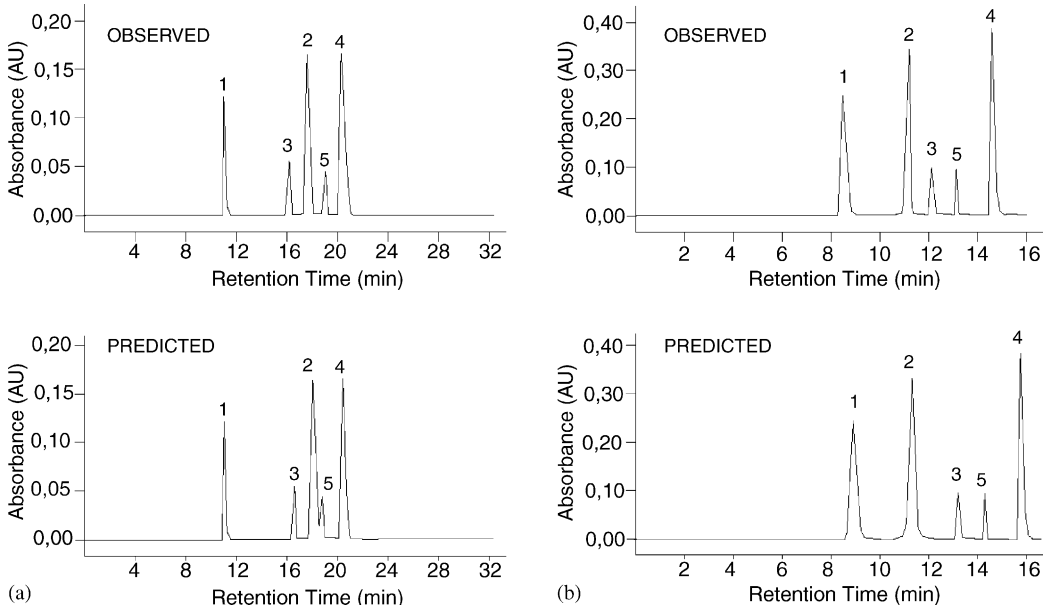

Fig. 4. Experimental and theoretically predicted chromatograms of test mixtures of analytes on XTerra MS C-18 column at the content of methanol in mobile phase of 3% (v/v) with the pH gradient time, t_G, of 25 min (a) and at the content of methanol 7% (v/v) with pH gradient time, t_G, of 20 min (b). $^{s}_{w}$pH ranged from 10.50 to 3.00. Analytes are: (1) aniline, (2) *p*-anisidine, (3) 2-amino-5-nitropiridyne, (4) 2-methylbenzimidazole, (5) morphine.

Table 2
Isocratic retention times (in min) of non-dissociated, $t_{[B]}$ and dissociated, $t_{[HB^+]}$ forms of test bases on XTerra MS C-18 column in relation to the content of methanol in the mobile phase

Analyte	% (v/v) MeOH							
	3%		7%		11%		15%	
	$t_{[B]}$	$t_{[HB^+]}$	$t_{[B]}$	$t_{[HB^+]}$	$t_{[B]}$	$t_{[HB^+]}$	$t_{[B]}$	$t_{[HB^+]}$
Aniline	11.87	2.12	8.67	1.76	6.72	1.65	5.44	1.60
p-Anisidine	18.80	2.32	11.41	1.84	7.79	1.56	5.76	1.49
2-Amino-5-nitropyridine	25.76	1.98	16.37	1.65	11.33	1.39	8.40	1.25
2-Methylobenzimidazole	36.80	4.75	23.09	2.96	16.00	2.13	11.76	1.73
Morphine	(74.04)[a]	3.36	42.08	2.05	22.61	1.12	13.89	1.09

In parenthesis are data calculated from the linear solvent strength theory on the basis of two initial methanol gradient runs of 5–100% (v/v) B at gradient times, t_G, of 20 and 60 min.
[a] Not determinable experimentally.

one (higher pH). Thus, the tail is consequently pushed back into main peak and peak tailing diminishes. In pH gradient HPLC peak compression might only be expected if analyte retention takes place at pH close to its pK_a. Otherwise, the analyte moves actually at isocratic conditions.

Based on the necessary experimental data, i.e., the retention times of dissociated and non-dissociated forms and the ${}^{s}_{w}pK_a$ (HPLC) calculated from a single pH gradient run (Tables 1 and 2) the expected pH gradient retention times can be calculated for different chromatographic conditions. In Table 3 the observed and the calculated retention times are collected. The two type of data agree well as evidenced by the low mean relative errors of the predictions. It is also clear that the model proposed follows the changes

Table 3
Experimental, $t_{R\,exp}$, and calculated, $t_{R\,calc}$, pH gradient retention times (in min) of test analytes on XTerra MS C-18 column for four concentrations of methanol in mobile phase and for various pH gradient times, t_G

(a) 3% (v/v) MeOH

Analyte	Gradient time, t_G																	
	7			10			15			20			25			30		
	$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using	
		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$
Aniline	8.65	8.39	8.58	10.80	10.27	10.49	12.00	11.74	11.79	11.73	11.86	11.86	11.68	11.87	11.87	11.71	11.87	11.87
p-Anisidine	8.85	8.66	8.62	11.15	10.86	10.80	14.16	14.16	14.07	16.29	16.75	16.67	17.65	18.22	18.17	18.29	18.66	18.64
2-Amino-5-nitropyridine	7.79	7.40	7.06	9.73	9.22	8.73	12.13	12.07	11.35	14.40	14.70	13.77	16.48	17.09	15.98	18.29	19.22	17.99
2-Methylo-benzimidazole	10.45	9.92	10.03	13.41	11.78	11.93	15.87	14.84	15.07	17.76	17.80	18.11	20.21	20.65	21.02	22.64	23.34	23.78
Morphine	9.47	8.40	7.70	11.97	10.17	9.16	14.35	13.05	11.54	16.55	15.86	13.85	18.91	18.59	16.10	21.49	21.24	18.27
Mean relative error (%)		5.31	7.10		7.99	10.16		3.64	6.69		2.08	5.22		2.49	5.29		2.55	4.99

(b) 7% (v/v) MeOH

Analyte	Gradient time, t_G											
	5			10			15			20		
	$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using		$t_{R\,exp}$	$t_{R\,calc}$ using	
		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$		${}^{s}_{w}pK_a$	${}^{w}_{w}pK_a$
Aniline	6.83	6.51	6.64	8.45	8.57	8.61	8.59	8.67	8.67	8.59	8.67	8.67
p-Anisidine	6.75	6.56	6.53	9.55	9.73	9.68	10.93	11.22	11.20	11.31	11.39	11.39
2-Amino-5-nitropyridine	6.05	5.76	5.51	8.75	8.62	8.14	10.56	11.10	10.44	12.27	13.15	12.37
2-Methylo-benzimidazole	7.33	7.03	7.10	10.03	10.17	10.33	12.51	13.12	13.35	14.75	15.81	16.10
Morphine	6.35	6.01	5.51	8.75	8.92	7.93	11.04	11.70	10.20	13.25	14.32	12.34
Mean relative error (%)		4.35	6.27		1.63	4.52		3.91	3.77		4.81	3.69

The retention times were calculated using either ${}^{s}_{w}pK_a$ (HPLC) parameters previously determined (Table 1) or ${}^{w}_{w}pK_a$ values taken from the literature [40–42].

Table 4
Gradient retention times, t_R, (in min) obtained in two methanol gradient HPLC runs of different gradient time, t_G at pH 10.50 for bases and 3.00 for acids, on XTerra MS C-18 column

No.	Analyte	Gradient retention time, t_R	
		t_G = 20 min	t_G = 60 min
Bases			
1	Aniline	6.24	7.79
2	*N*-Ethylaniline	7.52	10.99
3	Papaverine	8.48	14.11
4	Morphine	10.19	16.53
5	Brucine	12.64	25.41
6	Codeine	15.07	33.04
7	*p*-Anisidine	6.51	8.81
8	Acridine	8.83	13.81
9	*N*-Methylaniline	10.80	20.43
10	*N*-Benzodimethylaniline	12.35	23.01
11	2,4,6-Collidine	13,47	27.63
12	2,2′-Bipyridene	8,59	13.31
13	2-Amino-5-nitropyridyne	12,48	26.59
14	Benzylamine	13.97	31.81
15	*N*,*N*-Diethylaniline	16.99	38.72
16	2-Methylbenzimidazole	12.91	28.43
Acids			
1	Barbituric acid	9.84	17.96
2	Warfarine	15.05	34.77
3	1-Naphthylacetic acid	13.02	27.56
4	2,6-Dimethyl-4-nitrophenol	12.98	26.28
5	*p*-Nitrophenol	9.03	13.98
6	2-Chloro-4-nitrophenol	12.17	22.98
7	2,4,6-Trichlorophenol	15.64	34.77
8	*p*-Toluic acid	11.89	23.28

Methanol content, % B, changed from 5 to 100% (v/v).

Table 5
Contents of organic modifier (methanol), % B, in eluent and methanol gradient RP HPLC retention times, t_R, on Xterra MS C-18 column observed and calculated from the linear solvent strength theory

No.	Analyte	% B	$t_{R\,(observed)}$	$t_{R\,(calculated)}$
Bases				
1	Aniline	0.00	13.93 (±0.18)	11.64
2	*N*-Ethylaniline	30.00	13.15 (±0.71)	15.98
3	Papaverine	44.00	12.05 (±0.24)	15.08
4	Morphine	14.00	18.85 (±2.87)	15.56
5	Brucine	38.00	13.66 (±0.24)	16.10
6	Codeine	35.00	12.83 (±0.17)	16.19
7	*p*-Anisidine	3.00	15.49 (±0.33)	13.49
8	Acridine	46.00	14.68 (±0.17)	15.71
9	*N*-Methylaniline	19.00	12.83 (±0.11)	15.00
10	*N*-Benzodimethylaniline	38.00	12.58 (±0.08)	15.16
11	2,4,6-Collidine	34.00	12.72 (±0.10)	15.44
12	2,2′-Bipyridene	25.00	13.48 (±0.16)	16.08
13	2-Amino-5-nitropyridyne	7.00	14.47 (±0.06)	15.04
14	Benzylamine	12.00	13.28 (±0.18)	16.19
15	*N*,*N*-Diethylaniline	50.00	19.72 (±0.25)	23.55
16	2-Methylbenzimidazole	12.00	13.84 (±0.13)	15.21
Acids				
1	Barbituric acid	18.00	18.46 (±0.11)	20.00
2	Warfarine	46.00	18.54 (±0.19)	19.95
3	1-Naphthylacetic acid	34.00	18.82 (±0.17)	20.23
4	2,6-Dimethyl-4-nitrophenol	32.00	18.75 (±0.17)	19.93
5	*p*-Nitrophenol	8.00	20.50 (±0.20)	20.31
6	2-Chloro-4-nitrophenol	26.00	19.14 (±0.12)	20.36
7	2,4,6-Trichlorophenol	46.00	18.44 (±0.09)	20.46
8	*p*-Toluic acid	26.00	21.13 (±0.08)	21.50

In parenthesis are standard deviations.

in elution sequences wherever these are experimentally observed.

Studies on application of the pH gradient RP HPLC to the determination of pK_a started for a series of 24 test analytes with two organic modifier (methanol) gradient runs with different gradient times, t_G, at pH ensuring complete suppression of ionization. The t_G of 20 min and of 60 min was applied keeping the pH of 10.50, in case of bases, and of 3.00, when dealing with acid analytes. Retention times, t_R, measured in those gradient runs are collected in Table 4.

Based on the results of two initial organic gradient RP HPLC runs, for each test analyte that organic modifier (methanol) concentration was evaluated from the LSS theory [37], which provided retention times suitable for carrying out the pH gradient experiments. The retention time of about 15 min appeared to be the most suitable time for

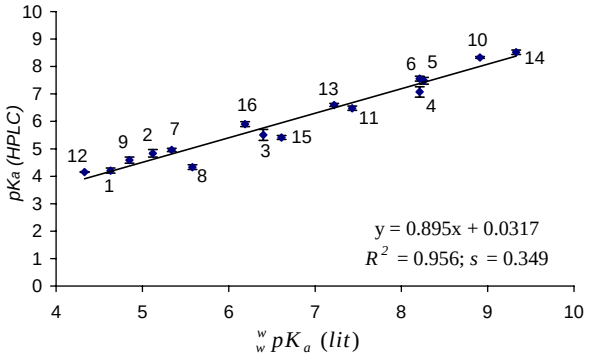

Fig. 5. Correlation, R^2, between $^{s}_{w}pK_a$ (HPLC) data determined by the pH gradient RP HPLC method on XTerra MS C-18 column and literature $^{w}_{w}pK_a$ data for a series of bases identified in Table 6. Bars denote standard deviations of measured data points and *s* is the standard error of estimate by regression.

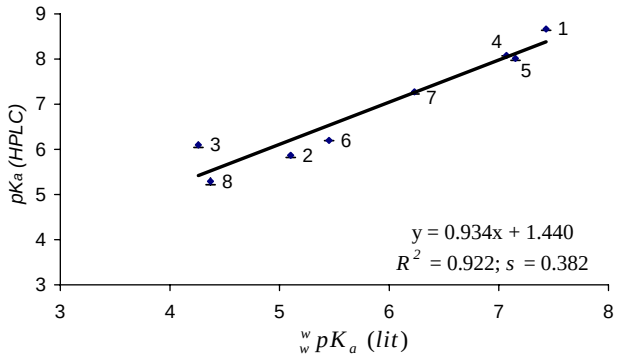

Fig. 6. Correlation, R^2, between $^{s}_{w}pK_a$ (HPLC) data determined by the pH gradient RP HPLC method on XTerra column and literature $^{w}_{w}pK_a$ data for a series of acids identified in Table 6. Bars denote standard deviations of measured data points and *s* is the standard error of estimate by regression.

Table 6
pH gradient RP HPLC retention times, t_R, obtained on XTerra MS C-18 column at specified times of gradient, t_G, along with the isocratic retention times of ionized forms of analytes, $t_{R\,ion}$, the reference acidity constants, ${}^{w}_{w}pK_a$ (lit), and the chromatographically determined acidity parameters ${}^{s}_{w}pK_a$ (HPLC)

No.	Analyte	t_R	$t_{R\,ion}$	${}^{w}_{w}pK_a$ (lit)	${}^{s}_{w}pK_a$ (HPLC)
Bases (t_G = 12 min)					
1	Aniline	12.91 (±0.43)	2.67 (±0.03)	4.63	4.21 (±0.09)
2	*N*-Ethylaniline	12.30 (±0.48)	1.70 (±0.05)	5.12	4.84 (±0.14)
3	Papaverine	10.95 (±0.29)	1.72 (±0.01)	6.40	5.51 (±0.20)
4	Morphine	9.71 (±0.58)	1.25 (±0.00)	8.21	7.07 (±0.19)
5	Brucine	8.71 (±0.38)	1.36 (±0.00)	8.26	7.48 (±0.12)
6	Codeine	8.39 (±0.41)	1.10 (±0.02)	8.21	7.56 (±0.09)
7	*p*-Anisidine	12.58 (±0.54)	2.72 (±0.07)	5.34	4.96 (±0.06)
8	Acridyne	13.65 (±0.60)	2.41 (±0.12)	5.58	4.33 (±0.08)
9	*N*-Methylaniline	12.18 (±0.32)	1.92 (±0.05)	4.85	4.59 (±0.11)
10	*N*-Benzodimethylaniline	7.12 (±0.33)	1.11 (±0.02)	8.91	8.33 (±0.03)
11	2,4,6-Collidine	9.98 (±0.41)	1.07 (±0.00)	7.43	6.48 (±0.08)
12	2,2′-Bipyridene	12.83 (±0.09)	3.24 (±0.16)	4.33	4.15 (±0.01)
13	2-Amino-5-nitropyridine	10.12 (±0.56)	1.91 (±0.03)	7.22	6.60 (±0.06)
14	Benzylamine	7.07 (±0.35)	1.65 (±0.03)	9.33	8.52 (±0.08)
15	*N*,*N*-Diethylaniline	13.14 (±0.87)	1.29 (±0.02)	6.61	5.41 (±0.07)
16	2-Methylbenzimidazole	11.10 (±0.56)	2.45 (±0.03)	6.19	5.90 (±0.09)
Acids (t_G = 15 min)					
1	Barbituric acid	14.85 (±0.06)	1.44 (±0.35)	7.43	8.67 (±0.03)
2	Warfarine	9.47 (±0.09)	1.56 (±0.02)	5.1	5.87 (±0.05)
3	1-Naphthylacetic acid	10.50 (±0.11)	2.58 (±0.03)	4.26	6.10 (±0.06)
4	2,6-Dimethyl-4-nitrophenol	13.94 (±0.06)	1.53 (±0.02)	7.07	8.08 (±0.01)
5	*p*-Nitrofenol	13.92 (±0.07)	1.23 (±0.00)	7.15	8.02 (±0.04)
6	2-Chloro-4-nitrophenol	10.44 (±0.08)	1.69 (±0.02)	5.45	6.20 (±0.01)
7	2,4,6-Trichlorophenol	12.55 (±0.08)	2.79 (±0.11)	6.23	7.27 (±0.05)
8	*p*-Toluic acid	8.82 (±0.19)	1.42 (±0.04)	4.37	5.29 (±0.07)

In parenthesis are standard deviations.

that. The requested methanol content in the mobile phase varied from 0% for aniline to 50% for *N*,*N*-diethylaniline. The organic gradient RP HPLC retention times, predicted and observed, are given in Table 5. The differences between the corresponding data are minor.

Now, having the earlier determined methanol concentration in the eluent, a programmed pH gradient RP HPLC experiment was carried out. The pH gradient run started at pH which ensured an effective suppression of ionization of analytes. It ended at pH providing analytes full ionization.

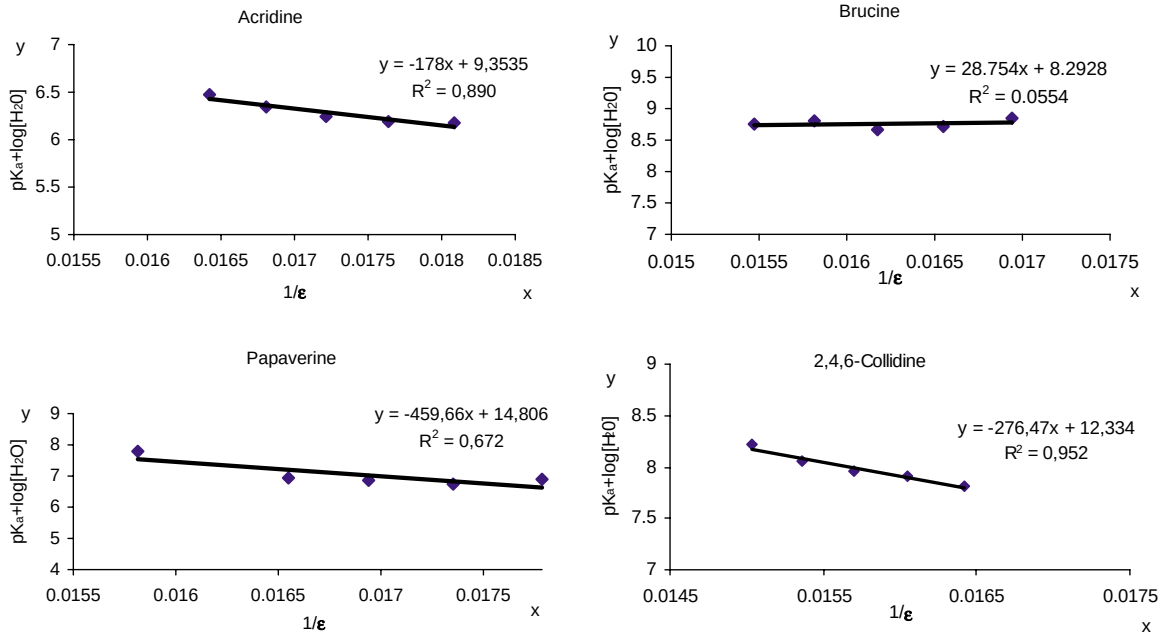

Fig. 7. Yasuda–Shedlovsky relationships for four exemplary basic analytes. Respective pK_a data were determined in pH gradient RP HPLC experiments.

Table 7
The ${}^{w}_{w}pK_a$ (HPLC) parameters determined by RP HPLC on XTerra MS C-18 column after Yasuda–Shedlovsky extrapolations

Analyte	${}^{w}_{w}pK_a$ (lit)	${}^{w}_{w}pK_a$ (HPLC)
2,4,6-Collidine	7.43	6.98
Acridine	5.58	5.29
Papaverine	6.40	7.07
Brucine	8.29	7.02

In practice, for bases the pH gradient starts at pH 10.50 and ends at pH 3.00. For acids, its starts at pH 3.00 and ends at pH 10.50.

Finally, at the pH ensuring a complete ionization of the analytes, an additional sample injection was made to get retention of the ionized forms of the compounds, $t_{R\,ion}$. Table 6 collects the pH gradient RP HPLC retention times, t_R, and the isocratically determined retention times of ionized forms, $t_{R\,ion}$, along with the determined, ${}^{s}_{w}pK_a$ (HPLC) and the literature, ${}^{w}_{w}pK_a$ (lit), dissociation constants.

The gradient times applied were evaluated from the retention times of non-ionized forms of analytes in a given chromatographic systems. Important was to ensure the gradient time shorter than the retention time, t_R, of the least retained analyte. Hence, the gradient time applied for bases was 12 min and for acidic analytes 15 min.

The pK_a values determined by pH gradient RP HPLC for a series of basic and acidic analytes strongly correlate with the reference ${}^{w}_{w}pK_a$ data taken from literature. The correlation coefficients, R, for basic and acidic analytes were 0.978 and 0.960, respectively (Figs. 5 and 6).

The effects of methanol content on ${}^{w}_{w}pK_a$ (HPLC) determined by the pH gradient method are given in Fig. 7. The Yasuda–Shedlovsky extrapolations [32,33] presented in Fig. 7 are assumed to eliminate the effects of organic modifiers on pK_a values. From Fig. 7 it can be seen that there really are more or less linear plots for pH gradient-determined ${}^{s}_{w}pK_a$ (HPLC) values. The ${}^{w}_{w}pK_a$ (HPLC) values obtained after Yasuda–Shedlovsky extrapolations (Table 7) are similar to the reference ${}^{w}_{w}pK_a$. Still, the ${}^{w}_{w}pK_a$ (HPLC) data differ for up to about 0.7 pK_a unit from the literature ${}^{w}_{w}pK_a$ data. These differences may be explained by specific inputs to the chromatographically determined parameters of specific interactions of analytes with silanol of the stationary phase material.

4. Conclusions

pH gradient HPLC is a new separation mode which extends analytical versatility of the technique. It appears to be especially suitable and effective for separation of ionogenic substances and may be a method of choice for separation of those bioanalytes which are sensitive to higher concentrations of organic solvents in eluents. If the retention of analyte takes place at actual pH gradient conditions ($t_d < t'_R < t_G + t_d$), the pH gradient mode produces narrow, symmetrical peaks of similar width, without tailing, what is an advantage over the standard isocratic mode of RP HPLC of ionizable analytes. Moreover, pH gradient run proceeds two to three times faster than conventional organic gradient HPLC.

pH gradient RP HPLC can be freely accomplished using regular equipment. Increasing availability of modern columns, which can be operated at a wide pH range makes the pH gradient HPLC even more attractive.

pH gradient RP HPLC can be comprehensively described in theoretical terms. The mathematical model here provided can be a good first approximation of real situation. It has successfully been verified empirically. Predictions from the model of the pH gradient retention times at different chromatographic conditions are acceptable. Hence, the rationale is provided to choose and to optimize separation conditions of analytes making use of only few predetermined experimentally data. The necessary empirical data are derived in three initial gradient HPLC runs: two organic gradient runs of different gradient time with pH of the buffer providing a full suppression of analytes' dissociation and one pH gradient run at a constant concentration of the organic modifier; at the end of that pH gradient run one additional injection provides the required for calculations isocratic retention time of a dissociated form of the analytes. From these data the values of ${}^{s}_{w}pK_a$ of analytes can be calculated and used for prediction of the pH gradient retention times at any chosen gradient times and also, for the prediction of isocratic retention at a given content of organic modifier and any pH.

pH gradient RP HPLC offers also a unique means to efficiently determine acidity of candidate compounds of desired biological or material properties. The chromatographically derived ${}^{w}_{w}pK_a$ (HPLC) parameters of both basic and acidic analytes strongly correlate to the reference acidity parameters, ${}^{w}_{w}pK_a$, so far arduously determined by the conventional titrations of aqueous solutions. The observed linear Yasuda–Shedlovsky relationships allow elimination of the effect of methanol, used as the organic modifier of the eluent, on the chromatographically determined pK_a. However, the reference and the chromatographically determined ${}^{w}_{w}pK_a$ parameters differ. Nonidentity of the chromatographic ${}^{w}_{w}pK_a$ parameters and the reference ${}^{w}_{w}pK_a$ data may not be disadvantageous from the medicinal chemistry view point as a two-phase HPLC system may better mimic the living system than the homogenous water solution. On the other hand, the chromatographically determined ${}^{s}_{w}pK_a$ values are the most appropriate for predicting effect of eluent pH on retention and hence for optimization of RP HPLC separation conditions.

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HPLC concept [13] presented in 2001 at the Symposium on Application of Theory to the Practice and Understanding of Chromatography, Ellecom, The Netherlands, honoring Dr. Lloyd R. Snyder. The project was financially supported by the Polish Committee for Scientific Research, Warsaw, Poland (Grant KBN No. 3PO5F02724).

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