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Modelling retention in liquid chromatography as a function of solvent composition and pH of the mobile phase

Rosa Bergés[1], Victoria Sanz-Nebot, José Barbosa*

Department of Analytical Chemistry, University of Barcelona, Avda. Diagonal 647, 08028 Barcelona, Spain

Abstract

The aim of this work was to develop a model that accurately describes retention in liquid chromatography (LC) as a function of pH and solvent composition throughout a large parameter space. The variation of retention as a function of the solvent composition, keeping other factors constants, has been extensively studied. The linear relationship established between retention factors of solutes and the polarity parameter of the mobile phase, E_T^N, has proved to predict accurately retention in LC as a function of the organic solvent content. Moreover, correlation between retention and the mobile phase pH, measured in the hydroorganic mixture, can be established allowing prediction of the chromatographic behavior as a function of the eluent pH. The combination of these relationships could be useful for modelling retention in LC as a function of solvent composition and pH. For that purpose, the retention behavior on an octadecyl silica column of a group of diuretic compounds covering a wide range of physico-chemical properties were studied using acetonitrile as organic modifier. The suggested model accurately describes retention of ionizable solutes as concomitant effects of variables included and is applicable to all solutes studied. We also aimed to establish an experimental design that allows to reproduce to a good approximation the real retention surface from a limited number of experiments, that is from a limited number of chromatograms. Ultimately, our intention is to use the model and experimental design for the simultaneous interpretive optimization of pH and proportion of organic solvent of the mobile phase to be used in the proposed separation.

Keywords: Retention modelling; Mobile phase composition; Diuretics

1. Introduction

Liquid chromatography (LC) is a routine method for solving many practical analytical problems. In addition to the broadening of its applications, advances have been made in our fundamental understanding of the separation mechanisms in LC. However, separations are still being developed in a non-systematic manner, often by trial-and-error, which involves several disadvantages. The most evident disadvantage is the long development time that is required to select experimental conditions that are not necessarily the optimum ones, and often are not as good as might be expected from this powerful technique.

The most important aspect of method development in LC is the achievement of sufficient selectivity (relative retention). Usually, the methods are focussed on optimizing the concentration of organic solvent in the mobile phase. However, the pH of the

*Corresponding author. Tel.: +34-93-4021-279; fax: +34-93-4021-213.

[1]Present address: Unitat de Recerca en Farmacologia, Institut Municipal d'Investigacció Mèdica (IMIM), Doctor Aiguader 80, E-08003 Barcelona, Spain.

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mobile phase is also a powerful parameter for optimizing selectivity when separating ionizable compounds. Variations in the mobile phase pH may easily lead to important variations in selectivity because the degree of ionization of solute, stationary phases and mobile phase additives (e.g., ion-pairing reagents) may be affected by the pH [1]. Therefore, the most useful way to maintain a reasonable k' range and to achieve a good selectivity for the resulting separation is to vary simultaneously the pH and the solvent strength of the mobile phase.

If selectivity optimization procedures are to be developed, a model to describe the chromatographic retention in LC (represented by the retention factor, k') as a function of the parameters to be optimized, pH and proportion of organic solvent is needed:

$$k' = F(a_{H^+}, \varphi) \tag{1}$$

where a_{H^+} is the hydrogen ion activity ($a_{H^+} = 10^{-pH}$) and φ the fraction of organic modifier in the mobile phase. Lopes-Marques and Schoenmakers [2] derived and evaluated several mathematical models, which combined effects of pH and percentage of organic solvent, using different equations for each of the single parameters to be optimized. They obtained the best results by assuming retention factors to vary quadratically with φ, and the equation derived contains nine coefficients and two variables. However, too many coefficients are undesirable because many chromatograms are required to their initial estimation. Moreover, the modelling function derived was proved to be remarkably insensitive to changes in the values of some of the nine coefficients, suggesting that they have no physical meaning. Models derived by first describing the retention factor as a function of pH and then assuming the coefficients in that equation to be a function of organic solvent proportion are preferred over models derived the other way around [2].

The theory for studying the pH dependence of chromatographic retention for ionizable solutes in liquid chromatography were proposed by Horváth et al. [3] and verified by different authors for different type of compounds [1,4–9]. The expression:

$$k' = \frac{k'_{HA} + k'_{A^-}\dfrac{K_a}{a_{H_m^+}\, y_{A_m^-}}}{1 + \dfrac{K_a}{a_{H_m^+} y_{A_m^-}}} \tag{2}$$

represents the variation of the retention factor for a weak monoprotic acid, HA, with the hydrogen ion activity in the mobile phase, $a_{H_m^+}$. The dissociation constant in the acetonitrile–water mixture used as mobile phase is represented by K_a; $y_{A_m^-}$ is the activity coefficient of the dissociated acid in the mobile phase that can be calculated by the classical Debye–Hückel equation; and k'_{HA} and k'_{A^-} are the two limiting retention factors of the undissociated and fully dissociated acid, respectively. For a weak monoprotic base, BH^+, a similar equation can be written:

$$k' = \frac{k'_{BH^+} + k'_B\dfrac{K_a\, y_{BH_m^+}}{a_{H_m^+}}}{1 + \dfrac{K_a\, y_{BH_m^+}}{a_{H_m^+}}} \tag{3}$$

where K_a is defined as the dissociation constant of the protonated base (BH^+) in the hydroorganic mixture used as mobile phase, and k'_{BH^+} and k'_B are the limiting retention factors of the protonated compound and the undissociated base, respectively. Eqs. (2) and (3) include the pH values in the hydroorganic mixture used as mobile phase instead of the traditionally values in water, and take into account the effect of activity coefficients [7].

Establishing the variation of the coefficients k'_{HA}, k'_{A^-}, k'_{BH^+}, k'_B and K_a in Eqs. (2) and (3) with the proportion of organic modifier, one can obtain a equation that express the retention factor for acidic and basic solutes as a function of both pH and concentration of organic solvent in the mobile phase. The dissociation constant, K_a, cannot be assumed to be independent of φ. The relationships between K_a for different type of compounds and the proportion of organic solvent have been extensively studied and are well known [10,11]. The variation of retention as a function of the solvent composition (keeping other relevant factors constant) has been extensively studied. The linear solvation energy relationship (LSER) formalism [12–14] have been widely used to predict retention in liquid chromatography. When a system with a fixed pair of solute and stationary phase is considered [15]:

$$\log k' = (\log k')_s + s\pi^*_m + a\beta_m + b\alpha_m \tag{4}$$

The independent term and the coefficients in Eq. (4) depend on solute and stationary phase parameters;

the solvatochromic π^*_m parameter evaluates solvent dipolarity/polarizability [16]; and the solvatochromic parameters α_m and β_m evaluate solvent hydrogen bond acidity [17] and solvent hydrogen bond basicity [18] of the mobile phase, respectively. Taking into account that β_m values for acetonitrile–water mixtures, used here as mobile phases, are nearly constant [19,20] and the observed correlation between the normalized Dimroth and Reichardt polarity parameter, E^N_T, [21] and π^* and α parameters, $E^N_T = 0.009 + 0.415\pi^* + 0.465\ \alpha$ [22], Eq. (4) can be reduced to the single solvent parameter-dependent expression:

$$\log k' = C + eE^N_T \quad (5)$$

The linear correlation between the chromatographic retention, represented by the logarithm of the retention factor, and the E^N_T polarity parameter have been used to predict the chromatographic behavior of different types of compounds as a function of the proportion of organic modifier in the eluent [15,23–26]. In acetonitrile–water mixtures rich in water this exists as a structural region without disruptions on the structure of water molecules. At percentages of acetonitrile–water of approximately 20% the structure of the mixtures changes and presents microheterogeneity. Eq. (5) fits data very well within each one of these structural regions, for most solutes over wide ranges of acetonitrile composition in the mobile phase [15] and provides a useful tool for predicting retention due to the good linearity obtained and because a suitable prediction of retention for a specific solute in a fixed stationary phase can be achieved from only two experimental measurements of k' at two different mobile phase composition within one of the structural regions of the acetonitrile–water mixtures [20,27]. Because of its accuracy and simplicity, we judged it to be the best available as descriptor of retention as a function of percentage of organic solvent in the mobile phase.

In this work, an experimental study was made in order to establish the effects of concomitant variations of pH and acetonitrile in the mobile phase on retention of a series of diuretic compounds covering a wide range of acid–base properties. These type of substances are widely used therapeutically in the treatment of congestive heart failure and hypertension [28], and are included in the list of compounds banned in sport by the Medical Commission of the International Olympic Committee [29]. We also aimed to establish an experimental design that allows reproducing to a good approximation the real retention surface from limited number of experiments, that is a limited number of chromatograms. Ultimately, our intention is to use the model and experimental design for the simultaneous interpretive optimization of pH and proportion of organic solvent of the mobile phase to be used in the proposed separation.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile for chromatography (Merck, Darmstadt, Germany) and water obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona) were used. Ammonium acetate, concentrated phosphoric acid, potassium bromide and potassium hydrogenphthalate (dried at 110°C before use) were all from Merck and analytical-reagent grade.

Acetazolamide, amiloride (hydrochloride), bendroflumethiazide, benzthiazide, bumetanide, canrenone (potassium salt), chlorothiazide, chlorthalidone, diclorfenamide, ethacrynic acid, furosemide, hydrochlorothiazide, spironolactone, triamterene and trichlormethiazide were purchased from Sigma Química (Alcobendas, Madrid, Spain). Molecular structures of these diuretics are shown in Fig. 1. Stock standard solutions of 1 mg/ml were prepared by dissolving the compound in a 50:50 (v/v) acetonitrile–water mixture except for triamterene and diclorfenamide, which were dissolved in mobile phase. All these solutions were stored in the dark at 4°C. Working solutions were daily prepared by 10-fold dilution of the stock standard solutions in mobile phase; stock solution of diclorfenamide was used without dilution.

2.2. Apparatus

The chromatographic equipment consisted of an ISCO (Lincoln, NE, USA) Model 2350 pump, an injector valve with a 10-μl sample loop and a variable-wavelength absorbance detector (V^4, ISCO) operating at 275 nm. The chromatographic system was controlled by ChemResearch™ Chromatographic Data Management Controller software (version 2.4)

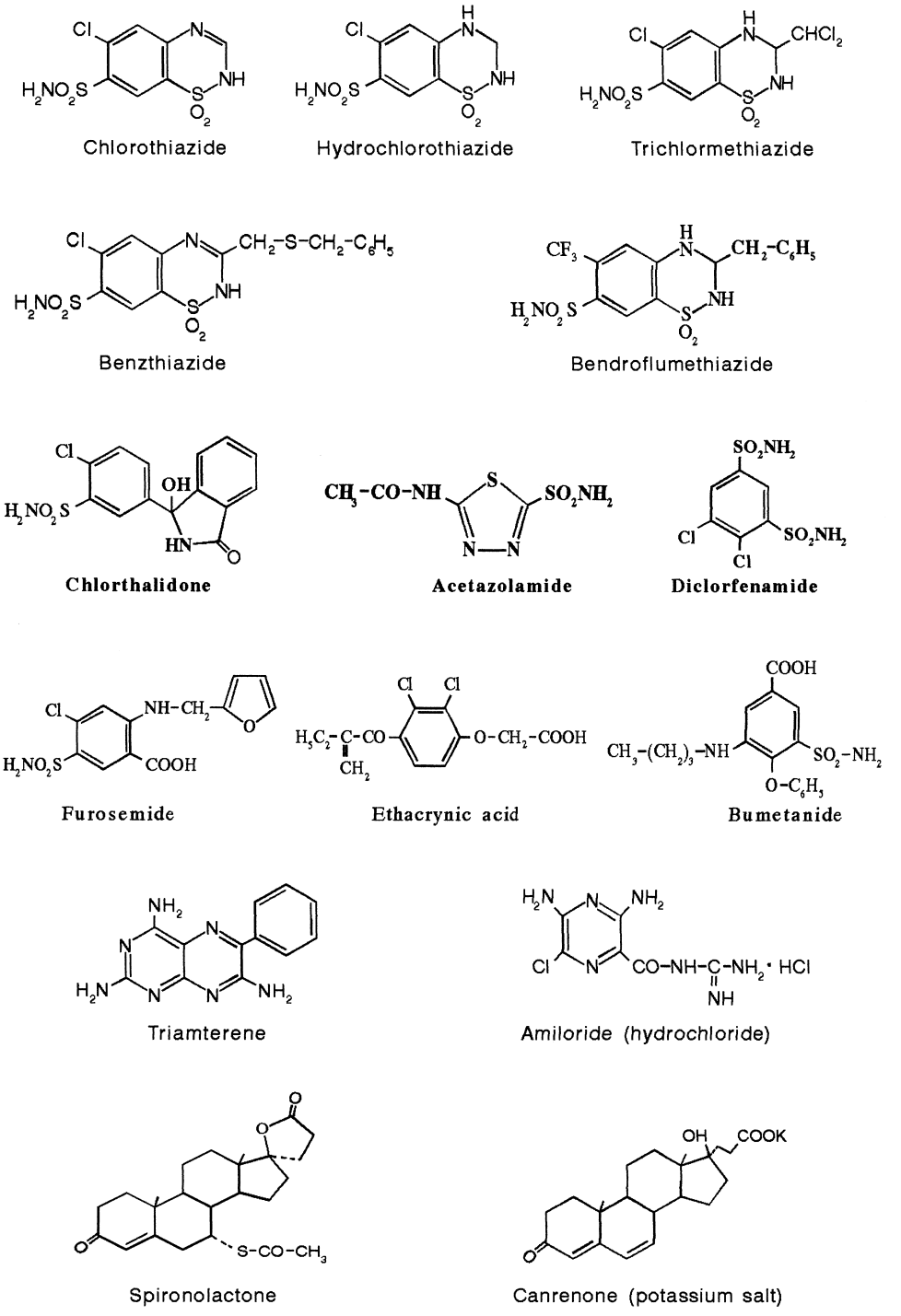

Fig. 1. Molecular structures for the diuretic compounds studied.

provided by ISCO. A 5-μm LiChrosphere 100 RP-18 (Merck) column (250×4 mm I.D.) was used at room temperature.

The e.m.f. measurements used to evaluate the pH of the mobile phase were done with a Model 2002 potentiometer (±0.1 mV) (Crison Instruments, Barcelona, Spain) using an Orion 8102 ROSS combination pH electrode (Orion Research, Boston, MA, USA). All solutions were thermostated externally at 25±0.1°C. The electrodes were stabilized in the appropriate acetonitrile–water mixtures prior to the e.m.f. measurements, which were performed in triplicate to ensure potentiometric system stability.

2.3. Chromatographic procedure

The concomitant effects of acetonitrile concentration and pH were studied for all solutes on the LiChrosphere column. Mobile phases used were mixtures of acetonitrile and a 0.1 mol/l ammonium acetate at different proportions of organic solvent, ranged from 10 to 60% (v/v). At each composition, different pH values were studied, spread over the pH range from 3 to 7, by adding concentrated phosphoric acid to the hydroorganic mobile phase. The aqueous component of the eluent was filtered through a 0.45-μm filter (MSI, Reactivos Scharlau, Barcelona, Spain) before use. Mobile phases were filtered through a 0.22-μm filter (MSI, Reactivos Scharlau), degassed under a stream of helium and the flow-rate was maintained at 1 ml/min.

The pH of the mixed mobile phases were measured using a 0.05-mol/kg potassium hydrogenphthalate solution as primary standard buffer reference, dissolved in the appropriate acetonitrile–water medium, and a combination pH electrode as described in previous works [15,30].

The hold-up time, t_0, was established for every mobile phase tested by injection of a 0.01% potassium bromide solution in water and monitoring the eluate at 200 nm [31]. The retention factors (k') for all the diuretic compounds at each mobile phase assayed were determined from four different injections of the diuretic compounds working solutions using the expression: $k' = (t_R - t_0)/t_0$, where t_0 is the hold-up time and t_R is the retention time of each compound obtained from the peak maximum. Solutes were injected individually to avoid mutual interferences.

Retention behavior for a group of compounds including acidic (furosemide, bumetanide, ethacrynic acid and canrenoic acid), weakly acidic (chlorothiazide and trichlormethiazide) and basic solutes (amiloride and triamterene) were used to assess the validity of the proposed model for substances with different acid–base properties.

All model-fitting calculations were performed with the SigmaPlot for Windows (version 4.00) curve fitter. This program uses the Marquardt–Levenberg algorithm to find the coefficients of the independent variable(s) that give the best fit between a given function and experimental data. The iterative algorithm seeks the values of the parameters that minimize the sum of the squared differences between the values of the observed and predicted values for the data until the convergence is achieved, that is differences between the residual sum of squares no longer decreases significantly.

3. Results and discussion

Acetonitrile–water mixtures are suitable for chromatography of neutral and weak acid diuretics [32], but for chromatography of basic and more acidic compounds control of the pH of the eluent by adding a phosphate buffer [32–34] is needed. Moreover, good peak symmetry of the basic diuretics is only obtained if an organic competing base [32,35,36] or an ammonium salt [37] is added to the acidic eluent. For these reasons we chose in our study a C_{18} stationary phase and a mobile phase consisting of acetonitrile and a 0.1 mol/l ammonium acetate solution, while the pH of the hydroorganic eluent was adjusted with concentrated phosphoric acid.

Retention factors (k') for the diuretic compounds were obtained at different percentages of acetonitrile in the mobile phase and at different pH, ranged from 10 to 60% (v/v) and from 3 to 7, respectively. Owing to the large retention times for spironolactone in mobile phases with percentages of acetonitrile below 35% (v/v), its retention factors have not been determined in the whole range studied.

The pH were measured in the hydroorganic mixtures using a 0.05 mol/kg potassium hydrogenphtha-

late solution as primary standard reference, dissolved in the appropriate acetonitrile–water medium and a combination electrode. These measurements can be performed as easily as in water as described in previous work [15,30] by the use of the operational definition of pH:

$$pH_X = pH_{PS} + \frac{E_{PS} - E_X}{g} \quad (6)$$

where pH_{PS} and E_{PS} are pH and electromotive force (e.m.f.) of the primary standard reference solution, pH_X and E_X are pH and e.m.f. of the mobile phase, and $g = 0.059$ V at 25°C. Rapid stabilization and good accuracy, precision and reproducibility for pH measurements up to 7 in acetonitrile–water systems are obtained [15]. pH measurements in mixed mobile phase permit the interpretation of chromatographic results without extrapolation of pH values from aqueous solutions.

According to Eq. (5), logarithms of the retention factors of the diuretic compounds studied correlate linearly with the polarity parameter of mobile phases containing 25–50% (v/v) acetonitrile with correlation coefficients higher than 0.99. On the other hand, the series of diuretics considered includes compounds with large differences in molecular structures (Fig. 1) and their chromatographic behavior as a function of the mobile phase pH depends on their acid–base properties. Depending on their acid–base behavior, diuretics can be classified into four groups [38]: basic (potassium-sparing diuretics, such as amiloride and triamterene), neutral (aldosterone antagonists, such as spironolactone), weakly acidic (carbonic anhydrase inhibitors, such as acetazolamide and diclorfenamide; thiazides and related compounds, such as chlorthalidone) and acidic compounds (canrenoic acid and loop diuretics, such as furosemide, bumetanide and ethacrynic acid). Neutral compounds are neither basic nor acidic and their retention is independent of pH. Weakly acidic compounds have sulphonamide groups with pK_a values higher than 7 [9,39,40] and all are uncharged in the pH range investigated and no significant effect of pH on retention factors are observed. According to their pK_a values [9,39,40] basic compounds present positively charged amine groups over the pH range studied and their chromatographic retention is low and also constant. The more acidic diuretic compounds have carboxylic acid groups with pK_a values between 3 and 6 depending on the compound [9,40], and plots of k' versus the pH showed the expected sigmoidal behavior [3]. Therefore, Eqs. (2) and (3) can be used to describe the retention behavior as a function of pH for all solutes considered. From the practical point of view, there is not a great influence of the activity coefficient values and these two equations can be simplified if activities are considered equal to concentrations.

Considering Eqs. (2) and (3), at each organic solvent concentration these equations describe a sigmoidal function. The curves are different for different organic solvent concentrations in mobile phase, because k'_{HA}, k'_{A^-}, k'_{BH^+}, k'_B and K_a vary with the percentage of acetonitrile in the mobile phase, but the general form of the equation is maintained. Establishing functions for the variations of the limiting retention factors and K_a with the proportion of organic modifier and substituting them into Eq. (2) (acidic compounds) or into Eq. (3) (basic solutes) we obtain a modelling function for k' as a function of both pH and concentration or organic solvent. Dissociation constants for some of the diuretic compounds studied have been potentiometrically determined in acetonitrile–water mixtures with concentrations of organic solvent up to 70% (w/w) [9]. Here we are assuming that K_a for solutes in the hydroorganic mixture constituting the mobile phase are known or that they can be estimated or extrapolated from the values in aqueous medium and the variation of K_a values of these type of substances in acetonitrile–water mixtures [42,43]. Otherwise, the number of coefficients to be adjusted in the general model derived should be increased.

k'_{HA} represents the retention factor of the protonated form of an acid, HA. Its variation with percentage of acetonitrile in the mobile phase, represented by the normalized Dimroth and Reichardt polarity parameter, should logically follow Eq. (5):

$$\log k'_{HA} = C_{HA} + e_{HA}E_T^N \quad (7)$$

and analogously for k'_{A^-}:

$$\log k'_{A^-} = C_{A^-} + e_{A^-}E_T^N \quad (8)$$

Substituting Eqs. (7) and (8) into Eq. (2) we obtain an expression that describes the variation of the

retention factors for acidic solutes as a combined function of pH and concentration of organic modifier in the mobile phase:

$$k' = \frac{10^{(C_{HA} + e_{HA} E_T^N)} + 10^{(C_{A^-} + e_{A^-} E_T^N)} \cdot K_a / a_{H_m^+} \, y_{A_m^-}}{1 + K_a / a_{H_m^+} \, y_{A_m^-}} \quad (9)$$

For a weak monoprotic base, BH^+, a similar equation can be obtained by substituting the expressions for variation of k'_{BH^+} and k'_B with E_T^N (analogous to Eqs. (7) and (8)) in Eq. (3):

$$k' = \frac{10^{(C_{BH^+} + e_{BH^+} E_T^N)} + 10^{(C_B + e_B E_T^N)} \cdot K_a \, y_{BH_m^+} / a_{H_m^+}}{1 + K_a \, y_{BH_m^+} / a_{H_m^+}} \quad (10)$$

where K_a is defined as the dissociation constant of the protonated base, and BH^+ and B represent the protonated compound and the undissociated base, respectively.

3.1. Retention modelling

Retention behavior for a group of compounds including acidic (furosemide, bumetanide, ethacrynic acid and canrenoic acid), weakly acidic (chlorothiazide and trichlormethiazide) and basic solutes (amiloride and triamterene) were used to assess the validity of the proposed model for substances with different acid-base properties.

The initial parameters values used by the curve fitter to find the equation model should be as close as possible to the real values because a good initial estimate guarantee better and faster results. The strategy to select the initial values for C_{HA}, e_{HA}, C_{A^-} and e_{A^-} in Eq. (9) is based on the fact that k'_{HA} and k'_{A^-} are the retention factors for the undissociated and fully dissociated acid. These parameters can be estimated from the experimental retention data corresponding to the highest and lowest pH values at every concentration of acetonitrile in the mobile phase for analytes with approximately pK_a values within 4 and 6, which constitute an estimation of k'_{HA} and k'_{A^-} values, and applying Eqs. (7) and (8). The same can be said for the estimation of initial values for basic compounds, C_{BH^+}, e_{BH^+}, C_B and e_B.

The coefficients found to describe the retention behavior of these solutes using Eqs. (9) or (10) are listed in Table 1, together with the sums of squares of the residuals (SSQ) and the relative root mean squared differences (RRMSD), to indicate the quality of the description of retention data. The SSQ is calculated from the differences between the observed retention factors and those calculated from Eqs. (9) (acid solutes) or (10) (basic compounds). In general, the small the SSQ the higher the fitness. By the nature of this definition solutes that show large retention factors, and thus large absolute deviations, tend to show the largest sums of squares. This is the reason why solutes such as ethacrynic acid and bumetanide show large values of the SSQ listed in Table 1. The RRMSD, obtained by calculating the

Table 1
Results of the application of the retention model proposed to all the experimental retention data available ($N > 40$) for each solute considered (coefficients describe chromatographic retention in terms of Eq. (9) for acids, and in terms of Eq. (10) for basic compounds)

Substance	C_{HA}	e_{HA}	C_{A^-}	e_{A^-}	SSQ[a]	RRMSD[b] (%)	N
Furosemide	−9.05	11.44	−10.06	11.57	2.24	10.1	45
Ethacrynic acid	−11.13	14.72	−14.40	17.23	65.73	13.9	46
Bumetanide	−13.08	16.90	−12.87	15.13	58.34	11.9	46
Canrenoic acid	−12.09	15.22	−12.18	14.20	4.12	7.3	49
Trichlormethiazide	−7.07	8.90	−6.29	5.91	1.38	7.4	45
Chlorothiazide	−3.93	4.39	−2.02	1.18	0.05	6.2	45
Substance	C_{BH^+}	e_{BH^+}	C_B	e_B	SSQ	RRMSD	N
Amiloride	−3.54	3.37	3.09	−2.21	0.06	15.9	45
Triamterene	−6.11	6.87	−5.91	7.11	0.12	11.0	25

[a] SSQ, sum of squares of the residuals $= \Sigma(k'_{obs.} - k'_{pred.})^2$.
[b] RRMSD, relative root mean squared differences $= 1000\sqrt{\Sigma(k'_{obs.} - k'_{pred.})^2 / \Sigma(k'_{obs.})^2}$

squared root of the SSQ divided by the sum of squares of the observed retention factors, provides the relative deviation (%) in the range studied and allows the comparison between different solutes presenting great differences in retention times. For most of the solutes the relative deviations were within a 6–14% margin. The only exceptions were the solutes with very small observed retention factors, such as amiloride, for which large contribution of retention factors around zero is observed.

The results shown in Table 1 demonstrate the good performance of the procedure. The iteration processes have a high convergence speed and are very robust because virtually the same final results are obtained from different guess initial values. Results were obtained using a relatively strict convergence criterion, the model was considered to have convergence if and only if the absolute value of the differences between the values of the residuals in consecutive iterations was less than 10^{-4}.

The retention of thiazide compounds, chlorothiazide and trichlormethiazide, as a function of pH remains almost constant as they are very weak acid, while the largest variations in retention are observed for more acidic compounds. Solutes with predominantly neutral behavior in the pH zone of interest were included in the fitting calculations for acidic compounds. There were two reasons to do so: first, to assess the behavior of the model for these compounds; second, if the models fit data for neutral solutes without problems, it will not be necessary to establish a rigorous threshold to distinguish between neutral, weak acidic and more acidic solutes.

The retention factor for the group of solutes studied presenting wide differences in their acid–base properties can be effectiveness described as a function of pH and acetonitrile content in the mobile phase with the equations proposed. However, as the retention factors of weakly acidic solutes are almost constant in the interval studied and their variations are mainly due to experimental error, some of the calculated parameters have no physical meaning and are just circumstantial. In these instances, k'_{HA} and k'_{A^-} have the same values, so that retention will be essentially independent of the value assigned to K_a.

Once the validity of the proposed model has been proved for solutes with great differences in physicochemical properties and in acid–base behaviors, it can be extended to the rest of diuretic compounds considered in this study. The model proposed can be used for the optimization of the mobile phase composition, acetonitrile content and pH, to be used for the chromatographic separation of these solutes in LC.

3.2. Experimental design

On determining a model to fit experimental data with the final objective of using it in a systematic method-development procedure, it is important to consider the number of experimental data points (chromatograms) required in order to reproduce to a good approximation the real retention surface. To assess the behavior of the model proposed when limited sets of data are available, we selected a prospective experimental design with four points (2×2) which corresponds to the highest and lowest pH values at every concentration of organic modifier. These points are: (pH, C) ((3, 30%), (3, 50%) (7, 30%) (7, 50%)). Next, we refitted the model for the 15 diuretic compounds considered using only the set of points defined by the experimental design. From the calculated coefficients, we evaluated the differences between the observed and the predicted re-

Table 2
Results obtained by fitting the model proposed, Eq. (9) for acidic and neutral compounds and Eq. (10) for basic solutes, to limited fraction of the data set (2×2 experimental design)

Substance	SSQ	RRMSD (%)	N
Furosemide	3.39	12.5	45
Ethacrynic acid	82.33	15.6	46
Bumetanide	82.11	14.2	46
Canrenoic acid	5.79	8.7	49
Amiloride	0.12	22.7	45
Triamterene	0.15	12.3	25
Chlorothiazide	0.11	9.7	45
Trichlormethiazide	2.06	9.0	45
Bendroflumethiazide	18.30	8.7	25
Hydrochlorothiazide	0.04	6.1	25
Benzthiazide	8.76	10.7	25
Chlorthalidone	0.08	5.6	25
Acetazolamide	9.1×10^{-3}	4.9	25
Diclorfenamide	0.31	7.7	25
Spironolactone[a]	108.07	15.6	20

[a] Data between E_T^N values from 0.84 to 0.80 (35–50%, v/v, acetonitrile).

tention values for all the data set. The results are given in Table 2 and they enabled us to obtain a rough idea of the degree of approximation for predictions made.

The initial values for the parameters C_{HA}, e_{HA}, C_{A^-} and e_{A^-} (acidic and neutral solutes) or C_{BH^+}, e_{BH^+}, C_B and e_B (basic solutes) can be estimated from the points of the experimental design, and applying Eqs. (7) and (8) or the equivalent equations for bases. The additional model fittings required for the initial-guess procedure do not cause a significant increase in the time required for the whole process because for the linear relationships described by Eqs. (7) and (8), a conventional linear regression method gives the result in a minimal amount of time. This small overhead in computation time is more than compensated by the increasing ease with which the whole process can be conducted. Dissociation constants for some of the diuretic compounds studied have been potentiometrically determined in acetonitrile–water mixtures with concentrations of organic solvent up to 70% (w/w) [9]. For the rest of solutes an estimation of the K_a in the acetonitrile–water mixtures constituting the mobile phase have been performed from values described in aqueous medium [39–41] and taking into account variations of this

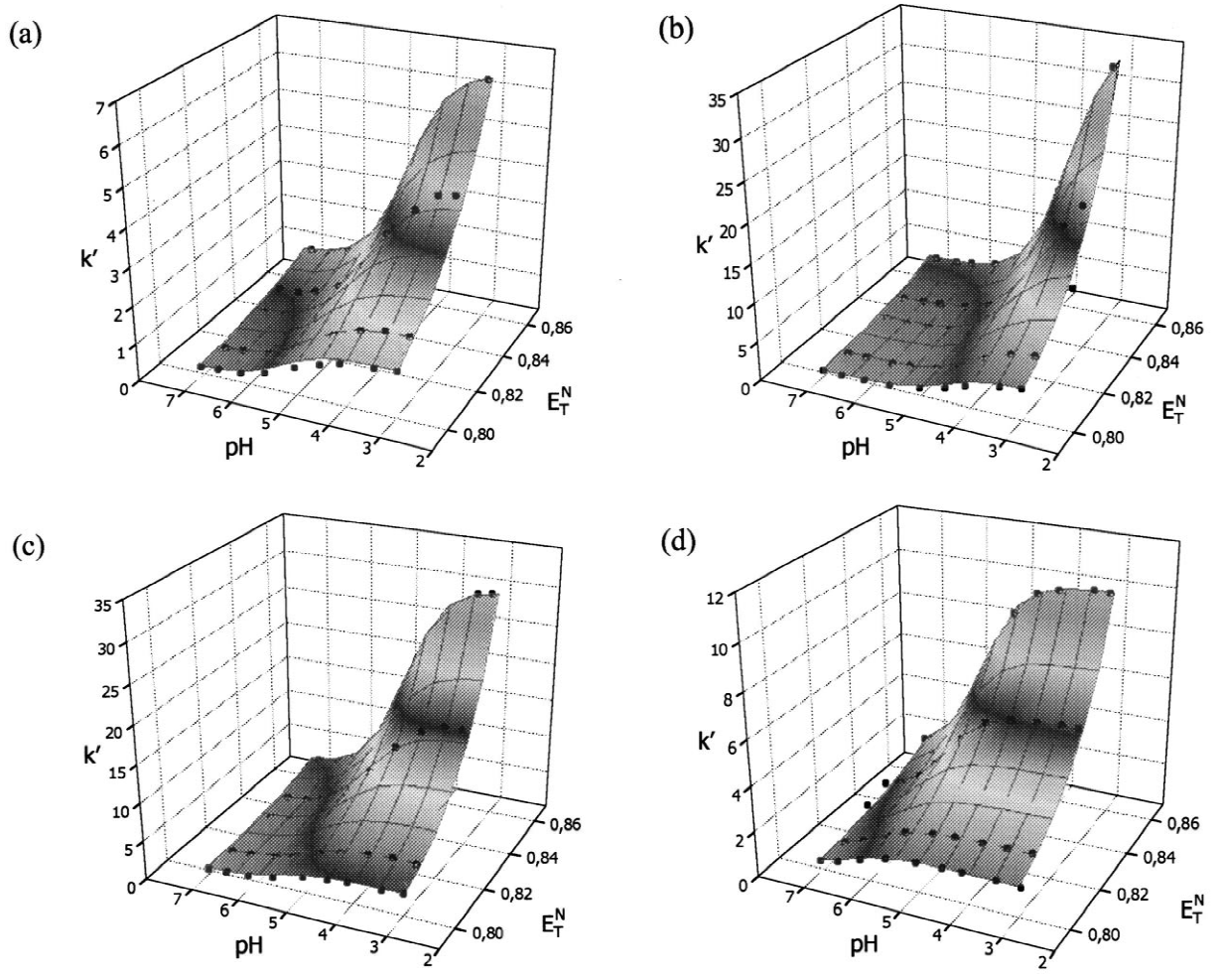

Fig. 2. Retention surfaces as a function of pH and the E_T^N polarity parameter of the mobile phase obtained by fitting the four data selected in the experimental design to Eq. (9). Solutes (acidic diuretics): (a) furosemide, (b) ethacrynic acid, (c) bumetanide and (d) canrenoic acid. Symbol: (●) experimental retention data over the whole parameter space.

dissociation constants when increasing acetonitrile concentration for similar compounds [9,42,43].

Figs. 2 and 3 show the retention surfaces for some of solutes studied, obtained by fitting four initial experimental data, as a function of pH and E_T^N polarity parameter of the mobile phase. Experimental retention data over the whole parameter space are also plotted together with the predicted surfaces to demonstrate the accuracy of the predictions done. The relative deviations at certain combination of pH and organic modifier concentration, where the retention factors are small, can be high. But, in general, the model gives very good results with relative deviations between experimental and predicted data from 5 to 15% for most of the solutes over the whole parameter space considered. The only exceptions are basic compounds such as amiloride and triamterene that not fit well to the general model of variation of k' values with pH (Eq. (3)) as it was found in a previous work [7]. Judging from the

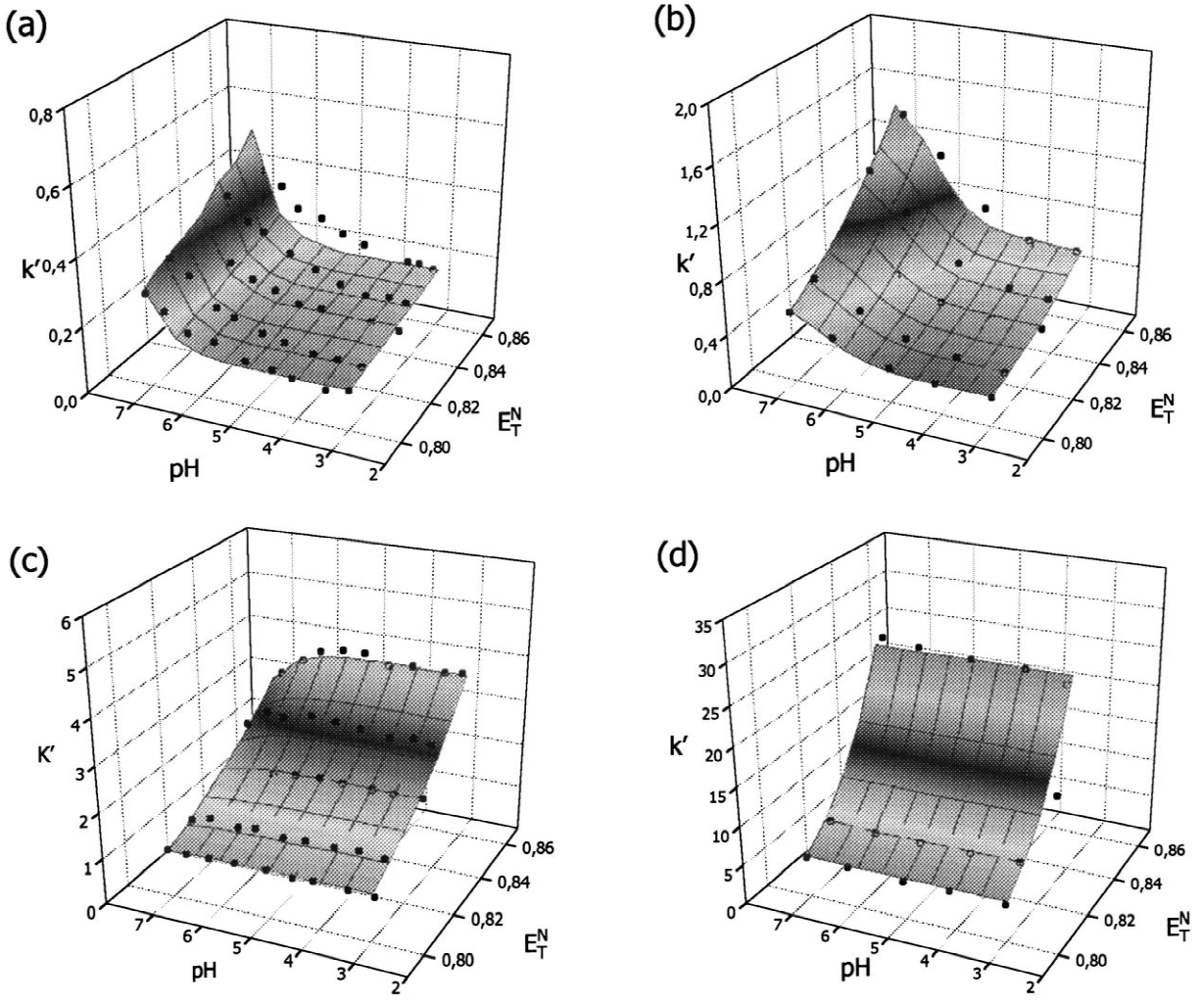

Fig. 3. Retention surfaces as a function of pH and the E_T^N polarity parameter of the mobile phase obtained: (1) by fitting the four data selected in the experimental design to Eq. (10): (a) amiloride, (b) triamterene (basic diuretics); (2) by fitting the four data selected in the experimental design to Eq. (9): (c) trichlormethiazide (weakly acid diuretic), (d) spironolactone (neutral diuretic). Symbol: (●) experimental retention data over the whole parameter space.

results using an experimental design of four points it is possible to predict retention factors for compounds with different acid–base properties accurately.

Our ultimate aim is not to describe retention, but to optimize separations. The model described by Eqs. (9) and (10) can be used to predict retention of diuretic compounds as a function of the acetonitrile composition and the pH of the mobile phase ranged from 30 to 50% (v/v) and from 3 to 7, respectively. From only four experimental data for each compound retention factors can be calculated over the whole parameters space studied and selectivity ($\alpha = k'_2/k'_1$) between adjacent solutes can be obtained. Three-dimensional graphs of the minimum selectivity (α_{min}) as a function of pH and concentration of acetonitrile are represented in Fig. 4. The highest minimum relative retention indicates the experimental conditions in which the optimum separation is obtained. The highest α_{min} value of 1.17 is predicted between triamterene and acetazolamide around 40% (v/v) acetonitrile ($E_T^N = 0.82$) in the mobile phase and at a pH between 3 and 3.5.

The chromatographic separation for the 15 compounds considered in a mobile phase containing 40% (v/v) acetonitrile and 60% (v/v) 0.1 mol/l ammonium acetate solution, to which concentrated phosphoric acid has been added to adjust the hydroorganic mobile phase pH to 3.5 is shown in Fig. 5. All solutes are well separated in an analysis time of 28 min with the exception of triamterene and acetazolamide for which an experimental selectivity of 1.10 is obtained. This little deviations from the predicted behavior can be due to the inaccuracies in the models for the basic diuretics [7]. Thus, the predicted composition of the mobile phase gives us a very good approximation to the experimental conditions to be used.

4. Conclusions

Chromatographic retention in LC can be described mathematically as a function of pH and solvent composition of the mobile phase. The retention factors modelling system developed by first describing the k' as a function of pH and then assuming the coefficients of this equation to be a function of composition proved to be very effective for compounds covering a wide range of acid–base properties. Moreover, the model proposed can be used for the optimization of the mobile phase composition because using a experimental design of four experimental data it is possible to predict retention factors accurately over the whole parameter space

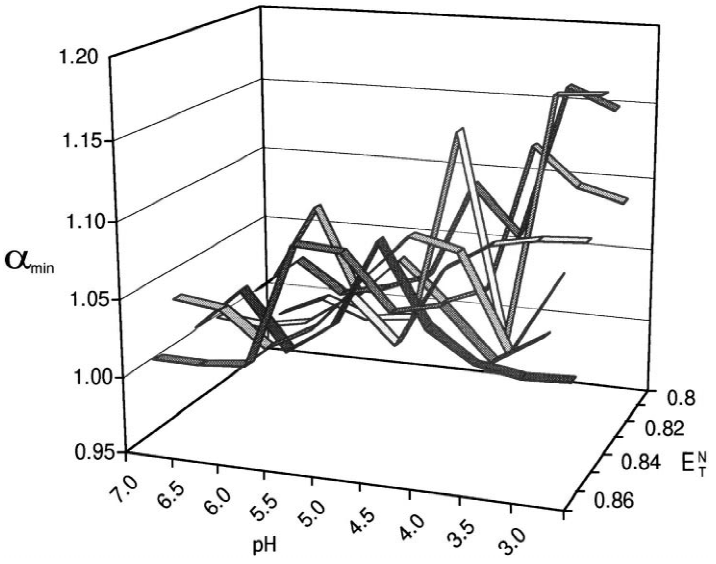

Fig. 4. Three-dimensional graph of minimum selectivity (α_{min}) as a function of pH and the E_T^N polarity parameter for the mixture of the 15 diuretic compounds studied.

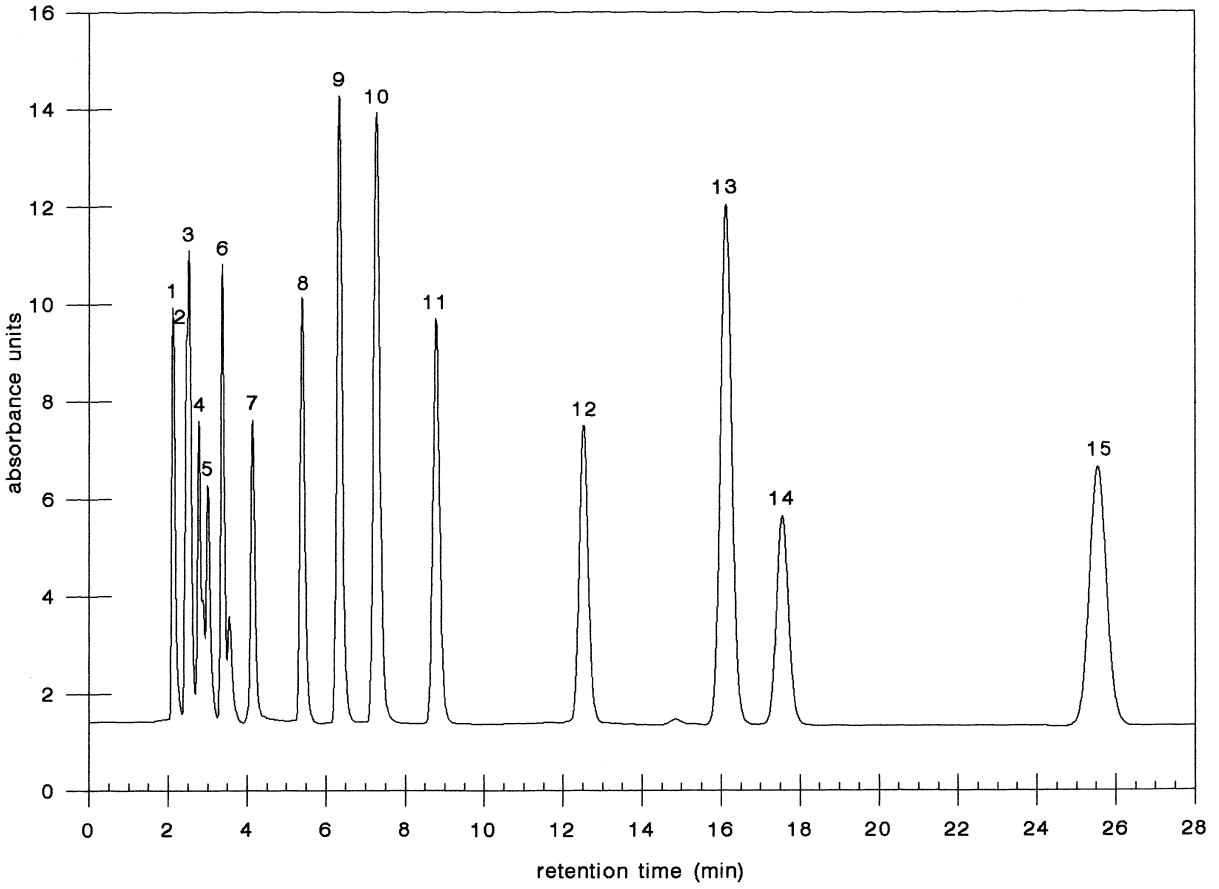

Fig. 5. Chromatogram obtained with the optimum mobile phase predicted. Peaks: (1) amiloride, (2) triamterene, (3) acetazolamide, (4) chlorothiazide, (5) hydrochlorothiazide, (6) chlorthalidone, (7) diclorfenamide, (8) trichlormethiazide, (9) furosemide, (10) canrenone, (11) benzthiazide, (12) bendroflumethiazide, (13) bumetanide, (14) ethacrynic acid and (15) spironolactone. See text for experimental conditions.

considered. We have incorporated the model derived into selectivity optimization procedures and it has proved to be effective and accurate.

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