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Retention behaviour of peptides, quinolones, diuretics and peptide hormones in liquid chromatography
Influence of ionic strength and pH on chromatographic retention☆

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Abstract

Peptides, quinolones, diuretics and peptide hormones are important substances of biomedical interest. In this work a model describing the effect of pH on retention in liquid chromatography (LC) is established and tested for these compounds using an octadecylsilica column. The suggested model uses the pH value measured in the hydro–organic mixture used as mobile phase instead of the pH value in water and takes into account the effect of the activity coefficients. The proposed equations permit the prediction of the pH optimum using a minimum number of measurements and also permit the determination of the acidity constants of the compounds considered in the medium used as mobile phase. Moreover, these equations can be combined with the previously derived equations, that relate the retention with the solvent composition of the mobile phase, to establish a general model that relates the elution behaviour of the solute with the significant mobile phase properties: composition, pH and ionic strength.

Keywords: Retention models; Dissociation constants; Ionic strength; Mobile phase composition; pH effects; Peptides; Quinolones; Diuretics; Peptide hormones

1. Introduction

Advances in biotechnology have provided the ability to prepare peptides and peptide hormones for therapeutic purposes. The use of peptides in biomedical therapy has increased in the last few years, because they have a large range of activity and specificity, usually with a low toxicity and a rapid metabolization [1,2]. However, during biosynthesis, impurities very close to the desired peptide will be present and may require separation and purification [2,3]. Thus, efficient separation of peptide and peptide hormones has become increasingly important for an ever-widening range of research disciplines in recent years. Likewise, quinolones comprise a relatively large and constantly expanding group of antibacterials widely used in clinical application and also widely applied in the treatment and prevention of veterinary diseases in food-producing animals.

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The presence of antibiotic residues in food gives cause for concern because of the emergence of resistant human pattogens and possible allergic hypersensitivity reactions in humans [4]. The European Union has established the maximum residue limits of veterinary products in foodstuffs of animal origin [3]. The fixing of these limits makes it essential to develop analytical methods capable of determining and confirming the presence of quinolone residues present in commercial products.

In order to test substances with wide differences in molecular structures and physico-chemical properties, a series of diuretics has been also included in this study. Diuretics are widely used therapeutically in the treatment of congestive heart failure and hypertension, among other diseases; moreover, have been misused in sport by competitors to reduce weight quickly, to dilute urine to prevent the detection of another groups and to control the retention of water produced by anabolic steroids; for this reason, diuretics are included in the list of compounds banned in sport by the Medical Commisssion of the International Olympic Committee since 1998 [6,7].

LC has proved very versatile in separation of different compounds from a great variety of sources [2–5,8–11]. Mobile phases containing mixtures of water and acetonitrile in a reversed-phase column has proved useful for solving most of the problems. Although a desired separation may be obtained by trial and error, this may take many attempts with subsequent loss of time and final substance yield, and could be a particular problem when only limited amounts of sample are available. We can minimize the total number of attempts by using experimental data to select the best conditions and by making use of accurate quantitative relationships able to predict elution of compounds under different separation conditions. Optimization of the chromatographic resolution of ionogenic solutes in LC is a task that is being actively researched. The two most useful optimization parameters are pH and the organic-modifier concentration. In previous works [12–15], an approach to optimizing the concentration of organic modifier in the mobile phase was tackled by stablishing relationships between retention parameters and Reichardt's E_T^N scale of solvent polarity [16]. The retention factors values, log k, of series of peptides, quinolones and diuretics [12–14] and the values of E_T^N parameter of the MeCN–water eluent system correlate linearly. This 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.

The inclusion of pH as an additional optimization parameter raises several problems [17]. The pH of the mobile phase is usually taken to be the same as that of the aqueous fraction. However, the pK_a values of the acids used to prepare the buffers change with the solvent composition [18,19] so does the pH of the buffer [20,21]. Sometimes the pH is measured after mixing the buffer with the organic modifier [22]. But even in this instance, the potentiometric system is usually calibrated with aqueous standards, and the measured pH is not the true pH of the mobile phase. Additionally, the proposed models do not consider the effect of the activity coefficients. This effect can be neglected in water, which has a high dielectric constant, but it may be considerable in MeCN–water mixtures [20].

pH measurements in the most widely used mobile phases, such as MeCN–water, methanol–water and tetrahydrofuran–water, can be preferred in a manner similar to that in water [23], taking into account the pH values previously assigned to primary standard buffer solutions in these media [21] according to the US National Institute of Standards and Technology (NIST) multiprimary standard scale. Also, in compliance with IUPAC rules [24,25], the activity coefficients of the species in hydro-organic mixtures can be calculated from the ionic strength through the classical Debye–Hückel equation [24,25].

In this work, through correct pH, pK_a and activity coefficient values a model describing the effect of pH on retention in LC is established and tested for series of peptides, quinolones, peptide hormones and diuretics covering a wide range of acid–base properties. The suggested model uses the pH value in MeCN–water mobile phases instead of pH value in water and takes into account the effect of activity coefficients. The usefulness of the proposed equations is twofold. They permit the prediction of the pH optimum for the separation methodologies using

a minimum number of measurements and also permit the determination of pK_a of analytes in the hydro-organic media used as mobile phases. Thus, these relationships may allow an improvement in chromatographic optimization schemes since drastically reduce the number of experimental data needed for developing a separation process. The proposed equations can be combined with the previously derived equation, that relate the retention with the solvent composition of the mobile phase [12–15], to establish a general model relating elution behaviour of the solutes with the significant mobile phase properties: composition, pH and ionic strength.

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm and acetonitrile (Merck, Darmstadt, Germany) were LC grade. Trifluoroacetic acid (TFA), sodium hydroxide, phosphoric acid, tetrabutylammonium hydroxide, potassium bromide and potassium hydrogenphthalate were all analytical grade obtained from Merck. Tripeptides used in this study were purchased from Sigma (St. Louis, MO, USA) and are Tyr–Gly–Gly, Ala–Leu–Gly, Gly–Gly–Ile and Gly–Gly–Phe. In the case of Ala–Leu–Gly, it was possible to separate two diastereoisomer mixtures named as Ala–Leu–Gly (1) and Ala–Leu–Gly (2). The selected peptide hormones studied were: oxytocin, bradykinin, Leu-enkephalin, triptorelin and buserelin. The peptide hormones were purchased from Sigma, except buserelin which is purchased from Hoechst Ibérica (Barcelona, Spain) and triptorelin which is purchased from Lasa (Barcelona, Spain). Tripeptides and peptide hormones were stored in a freezer at −4°C when not in use. The studied quinolones were obtained from various pharmaceutical firms: ciprofloxacin (Lasa), norfloxacin (Boral Química, Barcelona, Spain), enoxacin (Almirall, Barcelona, Spain), fleroxacin (Roche, Madrid, Spain), ofloxacin (Hoechst Ibérica) and pipemidic acid (Almirall and Prodesfarma, Barcelona, Spain). Ethacrynic acid, canrenoic acid (potassium salt), furosemide, bumetanide, triamterene and trichlormethiazide, were purchased from Sigma Química (Alcobendas, Madrid, Spain). The structures or sequences of the selected substances are shown in Fig. 1.

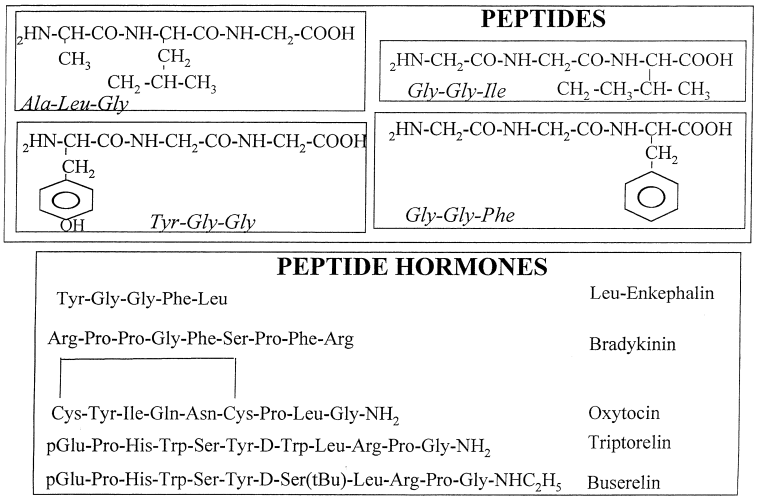
Stock solutions of the tripeptides and peptide hormones were prepared by dissolving approximately 10 mg of each substance and diluting to 5 ml; working solutions were prepared by 10-fold dilution of the stock solution. The mixtures of the tripeptides and peptide hormones studied was prepared dayly by 100-fold dilution of the stock solution. The solvent used as mobile phase was a mixture MeCN–water (7:93), 0.05% (v/v) TFA for tripeptides and a mixture MeCN–water (35:65), 0.1% (v/v) TFA for peptide hormones. The percentage of organic modifier in the mobile phase were previously optimized using linear solvation energy relationships [12].

Stock standard solutions of the quinolones were prepared in MeCN–water (10:90) at concentrations of 100 mg/l. A mixture of the six quinolones was prepared by diluting 5 ml of the ciprofloxacin, norfloxacin, fleroxacin and ofloxacin solutions, 2 ml of the enoxacin solution and 1 ml of the pipemidic acid solution to 25 ml with MeCN–water (10:90). The mobile phase used for quinolones study was MeCN–water (7:93), previously optimized [13].

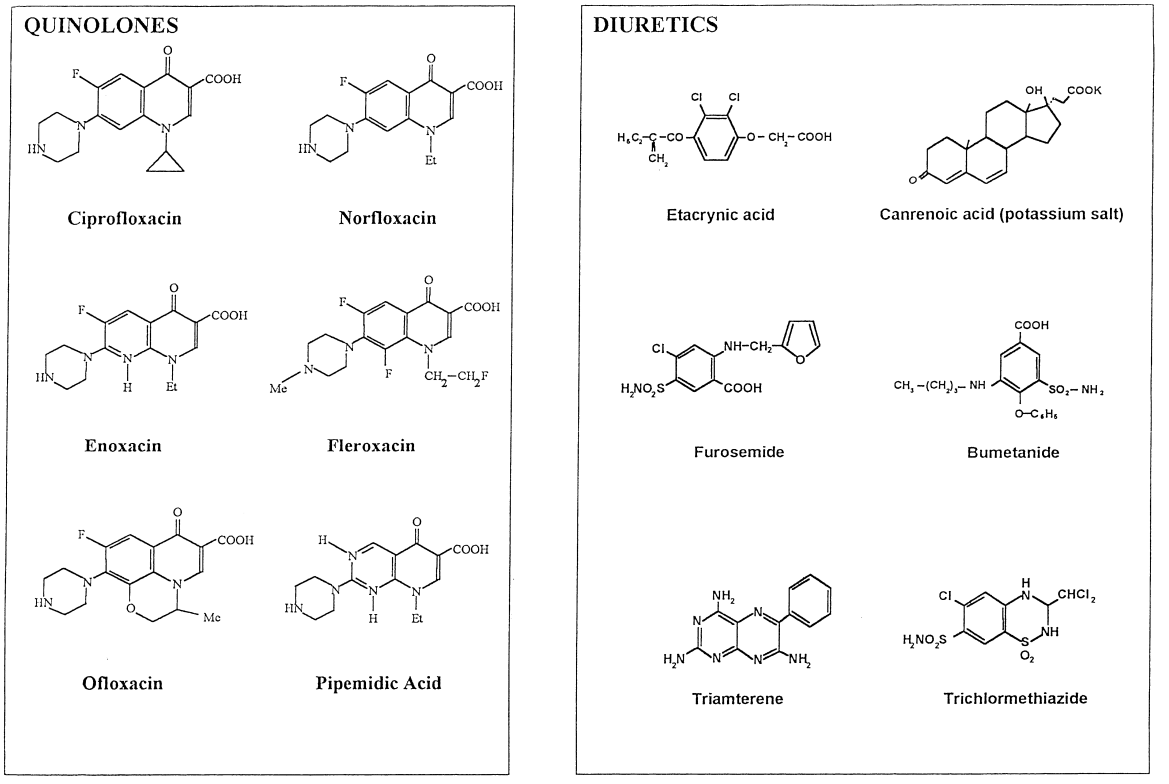
Stock standard solutions of diuretics were prepared by dissolving approximately 5 mg of each diuretic and diluting to 5 ml in a MeCN–water (50:50, v/v) mixture except for triamterene, which was dissolved in mobile phase owing to better solubility in this medium. Working solutions were prepared daily by ten-fold dilution of the stock standard solutions in mobile phase. This mobile phase was MeCN–water (40:60), previously optimized [14]. All the eluents and mobile phases were passed through a 0.22 μm nylon filter (MSI, Westboro, MA, USA) and degassed by sonication. The samples were passed through a 0.45 μm nylon filter (MSI).

2.2. Apparatus

The chromatographic equipment consisted of an ISCO Model 2350 (Lincoln, NE, USA) pump with an injection valve with a 10 μl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) operating at 214 nm (tripeptides and peptide hormones), 280 nm (quinolones) and 275 nm (diuretics).



(a)



(b)

Fig. 1. Structures of the substances studied.

The chromatographic system was controlled by ChemResearch Chromatographic Data Management System Controller Sotfware (ISCO) running on a Peceman AT Supermicro personal computer. A Merck LiChrospher 100 RP-18 (5 μm) column of 250×4 mm I.D. was used at room temperature.

The electromotive force (emf) values used to evaluate the pH of the mobile phase were measured with a potentiometer (±0.1 mV) Model 2002 (Crison Instruments, Barcelona, Spain) using an Orion 8102 ROSS combination pH electrode (Orion Research, Boston, MA, USA). All solutions were thermostatted externally at 25±0.1°C. The electrodes were stabilized in the appropriate MeCN–water mixtures before the emf measurements, which were performed in triplicate to ensure potentiometric system stability.

2.3. Chromatographic procedure

The mobile phase composition previously optimized was made of different acetonitrile–water mixtures: MeCN–water (7:93, v/v), 0.05% TFA (peptides), MeCN–water (35:65, v/v), 0.1% TFA (peptide hormones), MeCN–water (7:93, v/v) containing 25 m*M* phosphoric acid the aqueous fraction (quinolones), and MeCN–water (40:60) containing 0.1 *M* ammonium acetate the aqueous fraction (diuretics) [12–14].

In order to study the influence of the eluent pH on the chromatographic separation, the mobile phase was adjusted to different pH values, from 2 to 7, with sodium hydroxide (tripeptides and peptide hormones), tetrabutylammonium hydroxide (quinolones) and phosphoric acid (diuretics) using the percentages of acetonitrile previously optimized [12–14].

Retention factors were calculated from $k = (t_R - t_0)/t_0$, where t_0 is the retention time of the potassium bromide (hold-up time) which is established for each mobile phase composition and pH studied, and t_R is the retention time of each substance. The flow-rate of the mobile phase was mantained at 1 ml/min.

The pH was measured in the mixed mobile phase, where the chromatographic separation takes place, taking into account the reference pH values of primary standard buffer solutions, pH_S, for the standardization of potentiometric sensors in MeCN–water mixtures. This was assigned in previous works [20,21], in accordance with IUPAC rules [24,25] and on the basis of multiprimary standard scale, according to the NIST [23].

The knowledge of pH_S values allows to perform pH measurements in a mixed solvent as easily as in water taking into account the operational definition of pH [26,27]:

$$pH_X = pH_S + \frac{E_S - E_X}{g} \quad (1)$$

where E_X and E_S denote the emf measurements on the sample solution at unknown pH_X and on the standard primary reference solution at known pH_S, respectively, and $g = (\ln 10)\, RT/F$. For pH measurements we used as primary standard buffer reference solutions in the MeCN–water mixtures studied: phosphate buffer (0.030403 mol/kg Na_2HPO_4 and 0.008695 mol/kg KH_2PO_4) or potassium hydrogenphthalate (0.05 mol/kg) as standard buffer reference solutions.

The molar activity coefficients, *y*, were calculated using the classical Debye–Hückel expression:

$$\log y = \frac{-AI^{1/2}}{1 + a_0 BI^{1/2}} \quad (2)$$

where *A* and *B* are the Debye–Hückel constants and a_0 is the ion size parameter in the solvent mixture. These quantities have been reported previously [28]. The ionic strength, *I*, of the mobile phases used can be easily calculated for each pH value [29].

3. Results and discussion

The retention factor values, *k*, for the series of tripeptides, quinolones, peptide hormones and diuretics considered at different pH values of the mobile phase, were determined from three different injections at every mobile phase pH selected, and are shown in Figs. 2–5 respectively. Relative standard deviations lower than 5% for the *k* values were obtained. The percentage of acetonitrile in the hydro-organic mixture used as mobile phase, considered in this work, correspond to 7% (v/v) for the series of tripeptides and the series of quinolones, 35% (v/v) acetonitrile for the series of peptide hormones and 40% (v/v) acetonitrile for the series of diuretics. The

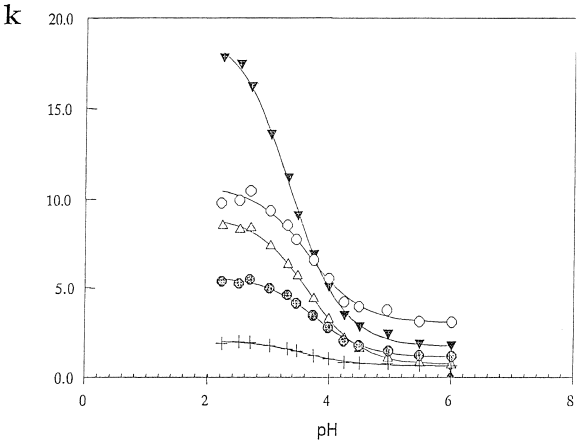


Fig. 2. *k* vs. pH for the tripeptides: ▼, Gly–Gly–Phe-; ○, Ala–Leu–Gly (2); △, Gly–Gly–Ile; ●, Ala–Leu–Gly (1); +, Tyr–Gly–Gly. Experimental conditions: MeCN–water (7:93, v/v), 0.05% TFA adjusting pH values up to 7 with sodium hydroxide.

optimization of mobile phase composition for the separation of the different series of substances was performed using the solvatochromic parameter E_T^N as solvent descriptor [16] and taking into account that log *k* values and E_T^N solvent parameter correlate linearly [12–15].

The pH values were measured in the aqueous–organic mobile phase, where the chromatographic separation takes place. The NIST recommends to choose standard reference solutions with pH_S values as close as possible to the unknown pH_x [23]. We use as standard reference solutions, the phosphate buffer and potassium hydrogenphthalate buffer, since these pH_S were previously determined in MeCN–water mixtures [20,21] and good accuracy and precision were obtained for pH values up to 7, using these reference pH_S values with commercial electrode [13]. In addition, rapid stabilization of the poten-

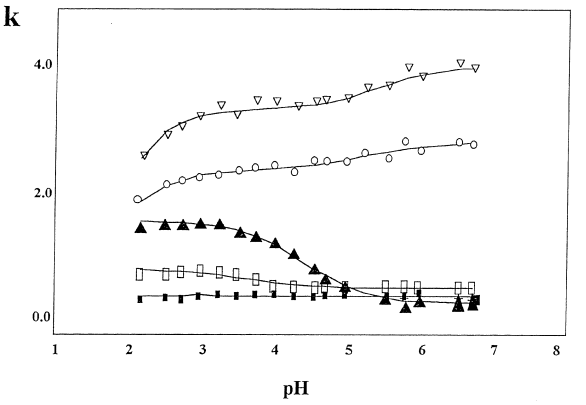


Fig. 4. *k* vs. pH for the peptide hormones: ▽, triptorelin; ○, buserelin; ▲, Leu-enkephalin; □, bradykinin; ■, oxytocin. Experimental conditions: MeCN–water (35:65, v/v), 0.1% TFA and adjusting pH values up to 7 with sodium hydroxide.

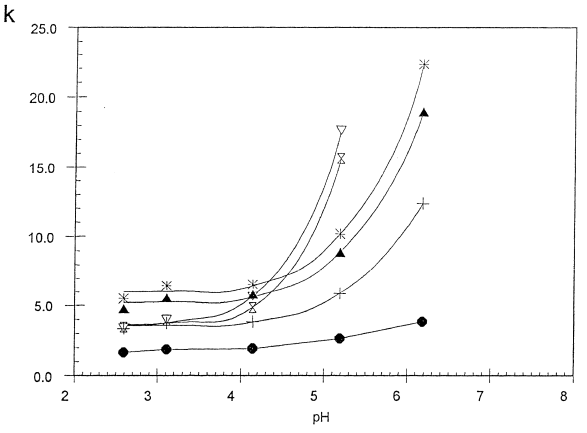


Fig. 3. *k* vs. pH for the quinolones: *, ciprofloxacin: ▲, norflacin, +, enoxacin; ▽, fleroxacin; ⧖, ofloxacin; ●, pipemidic acid. Experimental conditions acetonitrile–water (7:93, v/v), containing 25 m*M* phosphoric acid the fraction aqueous, adjusting pH up to 7 with sodium tetrabutylammonium.

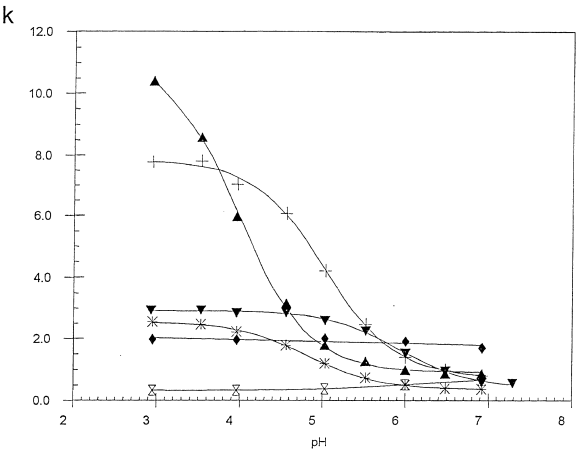


Fig. 5. *k* vs. pH for the diuretic acids; ▲, ethacrynic acid; +, bumetanide; ▼, canrenoic acid; *, furosemide; ⧖, triamterene; ♦, trichlormethiazide. Experimental conditions: MeCN–water (40:60, v/v) containing 0.1 *M* ammonium acetate the aqueous fraction and adjusting pH values up to 7 with phosphoric acid.

tiometric system was observed as shown in a previous work [13].

The octadecylsilica (ODS) stationary phase used, may only be used in the pH range 2–7, so it was no possible to study the retention of peptides, quinolones and peptide hormones as typical ampholytes, because correlation between *k* values and the pH of the mobile phase cannot be obtained over the entire range of pH. Thus, from a chromatographic point of view, with the widely used ODS stationary bonded phase, only the protolytic equilibria corresponding to pK_a values in the acid range are relevant.

The tripeptides studied usually have two relevant functional groups, Fig. 1. pK_a values in the acid range can be associated with carboxylic acid function and pK_a values in the basic range can be assigned to the protonated amino groups dissociation [30]. Thus, peptides can be considered as typical zwitterion forming compounds but only the acid–base equilibrium of the first functional group is within the interval of pH studied, Fig. 2.

Peptide hormones have different functional groups; in this way Leu-Enkephalin have one carboxylic acid C-terminal group, one group amino-N-terminal and one group phenol corresponding to the tyrosine residue; bradykinin have one carboxylic C-terminal group, one amino N-terminal and two guanidine groups corresponding to arginine residue; only pK_a associated to carboxylic group C-terminal are within the pH range studied, Fig. 4. Oxytocin have functional groups that are not within the pH range in study; for triptorelin and buserelin the group imidazol associated to residue of histidine is the only within the interval of pH considered.

The retention of peptides and peptide hormones is high at low pH values, Figs. 2 and 4, where the compound exists as a charged cation; when pH increases, the *k* value decreases, lewels off at isoelectric point pH and stays constant; this decrease in the chromatographic retention could be explained due to the equilibrium between the double charged zwitterionic and neutral forms is displaced to the first one. In the case of oxytocin, no variation of chromatographic retention when pH increases was observed and in the case of triptorelin and buserelin the *k* value increases slightly due to the small disociation of protonated basic groups when the pH increases.

The quinolones studied have two relevant acid–base equilibria within the pH range of physiological and pharmaceutical importance, due to the carboxylic and the ammonium groups of the piperazine ring [31–33]. pK_a values of the quinolones can be associated with carboxylic acid function [31–33], since this group is a stronger acid than the ammonium group. However, pK_a values for the quinolones were higher than those generally observed for carboxylic acid in hydro–organic media. This decrease in acidity was due to the formation of an intramolecular H-bond with the neighbouring keto function resulting in stabilization of the protonated species [31]. Since an ODS stationary phase was used, pH values in the acidic region were studied, and thus only the protolytic equilibria corresponding to pK_1 values of quinolones are relevant.

The retention of quinolones is low in strong acid media, Fig. 3, where the quinolone exist as single charged cation. As the pH increases, the *k* value increases. This one could be explained due to the equilibrium between the double charged zwitterionic and neutral forms is displaced to the latter form. This fact was comproved experimentally in a previous work, using a polystyrene–divinylbenzene column and adjusting the pH between 3.0 and 11.0 values [33]. This chromatographic behaviour is closely related to the physicochemical characteristics of the solute [34,35] and were observed only for zwitterionic substances with medium and high pK_1 constants [34] and with a difference between pK_1 and pK_2 of about two pH units [33]. Thus, this behaviour was not observed in studies concerned with variations in LC retention of the peptide with pH [36] because pK_1 of the peptides are about three units.

The series of diuretics considered includes compounds with large differences in molecular structures and their chromatographic behaviour depends on their acid–base properties. Chromatographic retention for weakly acidic (trichlormethiazide) and basic compounds (triamterene) is practically independent of pH in the range considered, Fig. 5. Weakly acidic diuretic compounds have sulfonamide groups with pK_a values higher than 7 [37] and are uncharged in the pH range investigated. According to their pK_a values [37], a basic compound like triamterene has positively charged amino groups over the pH range

studied and their chromatographic retention is low and also constant in acidic media. In Fig. 5, can be observed, however, that retention of triamterene is a little higher at neutral pH than in acidic media as correspond taking into account its pK_a values. The acidic compounds such as ethacrynic acid, furosemide and bumetanide have carboxylic acid groups with pK_a values between 3 and 6 depending on the compound, and plots of k versus the pH show the sigmoidal behaviour. The second protolytic equilibrium of furosemide and bumetanide is assigned to the dissociation of a sulfonamide group, with high pK_a values. At pH values below the carboxylic pK_a these compounds are uncharged and are more retained on the non-polar stationary phase used than at pH values above the pK_a where they are dissociated. Thus, only acidic diuretics vary their chromatographic retention significantly within the pH range studied and the optimum separation between these solutes will provide the optimum separation between all the diuretics considered.

Although the effect of solute ionization on retention are known, the theoretical interpretation of this phenomenon is hampered by the lack of a rigorous treatment of protolytic equilibria in hydro-organic mixtures. In doing this, pK_a values, pH, ionic strength, and the mean ionic activity coefficient, must be determined at each mobile phase composition studied after mixing the aqueous solutions with the organic modifier.

An equation which describes retention factor as a function of pH of the mobile phase, considering the activity coefficient, and accounts for every compound equilibrium that influences the retention in ODS columns, can be derived taking into account that the ionization in the mobile phase of peptides, quinolones and several peptide hormones in the mobile phase takes place according to the following equilibrium:

$$H^+BAH \Leftrightarrow H^+BA^- + H^+ \qquad K_a \tag{3}$$

where H^+BAH is the protonated form and H^+BA^- the zwitterionic form of the substances.

Equations that relate the retention of a compound in LC columns with the pH of the eluent, consider that the observed retention factor, k, is a weighted average of the k of the ionic and neutral forms of the solute [38], according to the molar fractions of these forms in the mobile phase. Thus the overall observed k values for the compounds considered can be given as:

$$k = x_{H^+BAH} k_{H^+BAH} + x_{H^+BA^-} k_{H^+BA^-} \tag{4}$$

where k_{H+BAH} and k_{H+BA-} are the retention factors and x_{H+BAH} and x_{H+BA-} the molar fraction of the protonated and zwitterionic forms of substances respectively.

Replacing the terms x_{H^+BAH} and $x_{H^+BA^-}$ by their expression for protolytes:

$$x_{H^+BAH} = \frac{a_{H^+}}{a_{H^+} + K_a y} \tag{5}$$

$$x_{H^+BA^-} = \frac{K_a y}{a_{H^+} + K_a y} \tag{6}$$

$$k = \frac{a_{H^+}}{a_{H^+} + K_a y} \cdot k_{H^+BAH} + \frac{K_a y}{a_{H^+} + K_a y} \cdot k_{H^+BA^-} \tag{7}$$

where K_a is the thermodynamic dissociation constant corresponding to the equilibrium (3).

Dividing by $K_a y$:

$$k = \frac{k_{H^+BAH} \cdot \dfrac{a_{H^+}}{K_a y} + k_{H^+BA^-}}{\dfrac{a_{H^+}}{K_a y} + 1} \tag{8}$$

The classic approach neglects activity coefficients and used the pH value in water instead of the pH value in the mobile phase.

For bradykinin, the pertinent equilibrium in the pH range studied correspond to the equilibrium:

$$H^{3+}BAH \Leftrightarrow H^{3+}BA^- + H^+ \qquad K_a \tag{9}$$

and the equation which relate the chromatographic retention and the pH of the mobile phase in this case can be derived in a similar way than Eq. (8) and is:

$$k = \frac{k_{H^{3+}BAH} \cdot \dfrac{a_{H^+} y^{2+}}{K_a y^{3+}} + k_{H^{3+}BA^-}}{\dfrac{a_{H^+} y^{2+}}{K_a y^{3+}} + 1} \tag{10}$$

In the cases of triptorelin and buserelin, the equilibrium to be considered in the acidic pH range is:

$$H^{2+}BAH \Leftrightarrow H^{+}BAH + H^{+} \qquad K_a \tag{11}$$

$$k = \frac{k_{H^{2+}BAH} \cdot \frac{a_{H^+} y}{K_a y^{2+}} + k_{H^+BAH}}{\frac{a_{H^+} y}{K_a y^{2+}} + 1} \tag{12}$$

In a similar way for acid diuretics the acid–base equilibrium correspond to the dissociation of a carboxylic acid:

$$HA \Leftrightarrow A^{-} + H^{+} \qquad K_a \tag{13}$$

$$k = \frac{k_{HA} + k_{A^-} \cdot \frac{K_a}{a_{H^+} y}}{\frac{K_a}{a_{H^+} y} + 1} \tag{14}$$

The usefulness of Eqs. (8), (10), (12) and (14) is twofold. They can be effectively used to calculate the pK_a values of the substances and the retention factors of the different species of the compounds considered, from the measured k value and the pH and y variables. But also, if pK_a of the substance can be estimated these equations permit the prediction of the optimum eluting pH conditions from a minimum number of experimental k–pH measurements.

The pK_a values of tripeptides, several peptide hormones and diuretic acids were determined from the experimental k and pH values and calculated y data, by a non-linear least-squares fit of the data to Eqs. (8), (10), (12) and (14). The obtained values are given in Table 1. Also in this Table are shown the potentiometric pK_a values previously determined [32,37] for peptides, quinolones and diuretics. By fitting the obtained data in above-mentioned equations, the solid lines showed in Figs. 2–5 were obtained. The figures show the perfect agreement between experimental and calculated k values. Various authors [39–41] have remarked on the advantages of the LC method for evaluating the ionization constants of substances. Small quantities of compounds are required, poor water-solubilitiy is not a serious drawback and the samples need not to be pure and that the purity of the substance is not a critical factor if impurities can be separated from the substance studied on the LC column. Experimentally determined chromatographic pK_a values, given in Table 1, were confirmed using potentiometric, electrophoretic and spectrophotometric methods in the same solvent composition used in the LC method [42–44]. It is seen that the chromatographic pK_a values, listed in Table 1, are in good agreement and randomly deviate from each other about 5% [44].

Table 1
pK_a values of peptides, quinolones, peptide hormones and diuretic drugs obtained from potentiometric (method 1)[a] and chromatographic (method 2)[a]

MeCn (%, v/v)	Substance	Method 1	Method 2
7	*Peptides*	pK_{a1}	pK_{a1}
	Tyr–Gly–Gly	3.45(0.01)	3.54(0.05)
	Ala–Leu–Gly (1)	3.57(0.02)	3.71(0.02)
	Ala–Leu–Gly (2)	3.57(0.02)	3.61(0.05)
	Gly–Gly–Ile	3.54(0.03)	3.59(0.02)
	Gly–Gly–Phe	3.21(0.02)	3.30(0.02)
35	*Peptide Hormones*		
	Leu-enkephalin	–	4.38(0.04)
	Bradykinin	–	3.21(0.14)
	Triptorelin	–	4.71(0.11)
	Buserelin	–	5.11(0.11)
7	*Quinolones*		
	Ciprofloxacin	6.13(0.05)	–
	Norfloxacin	6.26(0.05)	–
	Enoxacin	6.20(0.02)	–
	Fleroxacin	5.71(0.02)	–
	Ofloxacin	6.20(0.03)	–
	Pipemidic acid	5.58(0.03)	–
40	*Diuretics*		
	Ethacrynic acid	4.23	4.08(0.05)
	Canrenoic Acid	–	6.03(0.07)
	Furosemide	5.02	4.93(0.06)
	Bumetanide	5.27	5.13(0.07)
	Triamterene	6.26	–
	Trichlormethiazide	8.09	–

[a] Values in parentheses are standard deviations.

On the other hand, Eqs. (8), (10), (12) and (14) can be written in a linearized form:

$$\boldsymbol{k} \cdot \left(\frac{\boldsymbol{a_{H^+}}}{\boldsymbol{K_a y}} + \boldsymbol{1} \right) = k_{H^+BAH} \cdot \frac{\boldsymbol{a_{H^+}}}{\boldsymbol{K_a y}} + k_{H^+BA^-} \tag{15}$$

For bradykinin,

$$\boldsymbol{k} \cdot \left(\frac{\boldsymbol{a_{H^+} y^{2+}}}{\boldsymbol{K_a y^{3+}}} + \boldsymbol{1} \right) = k_{H^{3+}BAH} \cdot \frac{\boldsymbol{a_{H^+} y^{2+}}}{\boldsymbol{K_a y^{3+}}} + k_{H^{3+}BA^-} \tag{16}$$

For triptorelin and buserelin,

$$k \cdot \left(\frac{a_{H^+} y}{K_a y^{2+}} + 1 \right) = k_{H^{2+}BAH} \cdot \frac{a_{H^+} y}{K_a y^{2+}} + k_{H^+BAH} \quad (17)$$

For acid diuretics,

$$k \cdot \left(\frac{K_a}{a_{H^+} y} + 1 \right) = k_{A^-} \cdot \frac{K_a}{a_{H^+} y} + k_{HA} \quad (18)$$

Then, when pK_a values of substances are known, plots of the bold terms can be used in order to optimize the pH of the mobile phase from only two experimental k values at two $a_H \cdot y$ different values. Fig. 6 shows the corresponding plots for (a) several peptides, quinolones and peptide hormones (b) several peptide hormones as triptorelin, buserelin and bradykinin and (c) the diuretic acids studied. These plots also permit to obtain k values of the acid, zwitterionic or basic species of the compounds from the intercept and slope.

Having verified the linearity of Eqs. (8), (10), (12) and (140, it should be very useful in practice taking into account the large number of pK_a values that are known in MeCN–water mixtures [42]. Also, estimation of pK_a values in MeCN–water mixture can be possible, if pK_a values in water are known, taking into account the preferential solvation by water in these mixtures [45]. These equations can be applied to other compounds, whose pK_a values are known, and only two experimental values at two different pH values would be needed for the optimization of the pH of the mobile phase.

In order to examine the accuracy of retention, predictions obtained using these linearized equation, data measured for peptides and peptide hormones at pH 2.5 and 6, for quinolones at pH 3.11 and 5.19 and for diuretics at pH 2.95 and 5.52 in MeCN–water mixtures were considered. From just these two measurements per compound, k values of compounds considered at all different pH values were calculated by Eqs. (8), (10), (12) and (14) taking into account pK_1 values. Thus, selectivity was obtained for solute adjacent pairs in the usual way $\alpha = k_2/k_1$. As example, in Fig. 7 are shown the obtained α values for the series of peptides and for the series of diuretics. In Fig. 7, the solid lines indicate α values obtained from two measurements by compound

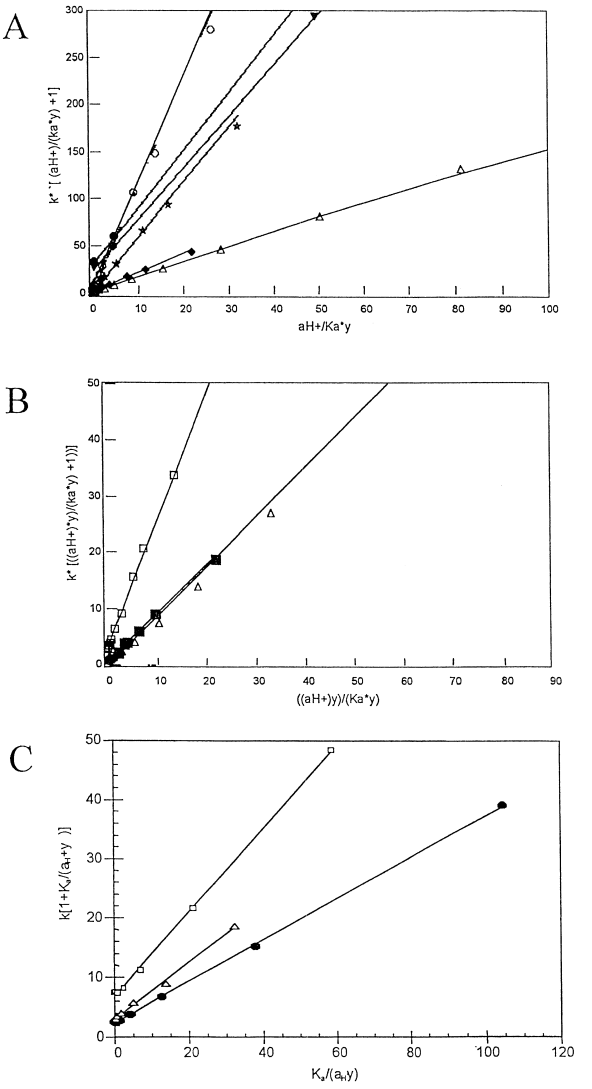


Fig. 6. Plots of k $(1+a_{H+}/K_a y)$ vs. $a_{H+}/K_a y$ for (A) ○, Ala–Leu–Gly (2); ●, ciprofloxacin; ▼, norfloxacin; ☆, Ala–Leu–Gly (1); ♦, Tyr–Gly–Gly; △, Leu-enkephalin. Plots of $(1+a_{H+}/K_a y^{3+})$ vs. $a_{H+} y^{2+}/K_a y^{3+}$ for (B): ■, brakykinin; □, buserelin; △, triptorelin. Plots for $(1+K_a/a_{H+} y)$ vs. $K_a/a_{H+} y$ for (C): □, bumetanide: △, canrenoic acid; ●, furosemide.

using linearized equations and the points shown are α values obtained from experimental. The concordance between both sets of values indicates that only two experimental measurements per compound are enough to predict accurately the effect of pH and

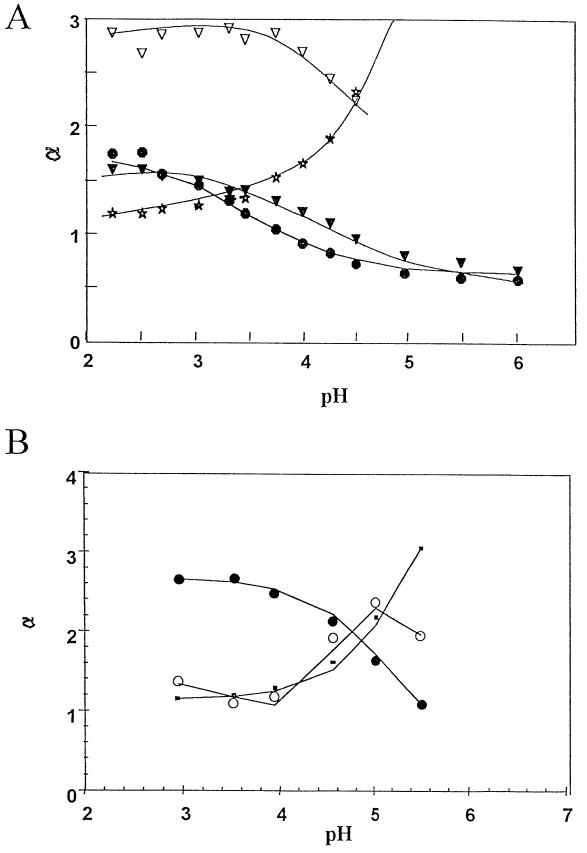


Fig. 7. Variation in selectivity (α values) for solute pairs with mobile phase pH. Solid lines indicate α predicted by Eqs. (8), (10), (12) and (14) from two measurements per compound. Symbols represent experimental α values for solute pairs: (A) ▽, Ala–Leu–Gly 1/Tyr–Gly–Gly; ▼, Gly–Gly–Ile/Ala–Leu–Gly 1; ☆, Ala–Leu–Gly 2/Gly–Gly–Ile; ●, Gly–Gly–Phe/Ala–Leu–Gly 2. (B) ●, bumetanide/canrenoic acid; ■, ethacrynic acid/bumetanide; ○, canrenoic acid/furosemide.

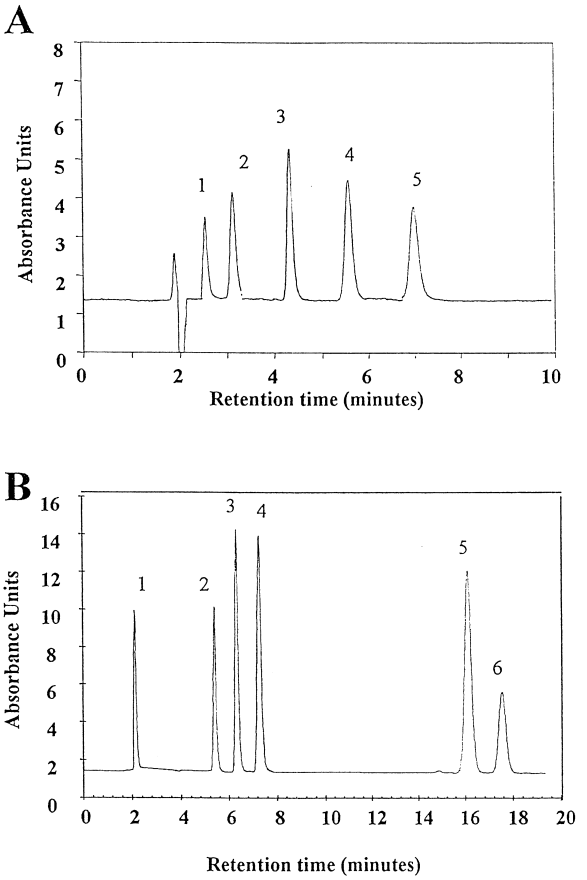


Fig. 8. Chromatogram of (A) peptide hormones at pH 3.3 (35% MeCN, 0.1% TFA): 1, oxytocin; 2, bradykinin; 3, Leu-enkephalin; 4, triptorelin; 5, buserelin. (B) Diuretics at pH 3.5 (40% MeCN, 0.1 *M* ammonium acetate in the aqueous fraction): 1, triamterene; 2, trichlormethiazide; 3, furosemide; 4, canrenoic acid; 5, bumetanide; 6, ethacrynic acid.

ionic strength in the chromatographic behaviour of the substances.

From results of selectivity, good chromatographic separation with a reasonable retention time can be obtained: for tripeptides at a pH of the hydro-organic mixtures between 2.5 and 3 and for quinolones between 3 and 4; for peptide hormones the best separation can be obtained at a pH of the hydro–organic mixtures between 3 and 3.5 and for diuretics at a pH of the hydro–organic mixtures between 3.5 and 4.5. Fig. 8 shows, as an example, the chromatogram of the separation of the substances studied following the optimal experimental conditions: for peptide hormone at pH 3.3 (35% MeCN) and for diuretics at pH 3.5 (40% MeCN).

From this study, it can be concluded that the use of the proposed models to relate the retention of ionizable compound with the pH of the mobile phase may be of considerable use in the optimization of chromatographic methods. The obtained relationships can be combined with those that relate the retention with the solvent composition of the mobile phase [12–15] in order to establish a general model

relating elution behaviour of the solutes with the composition, pH and ionic strength of the mobile phase.

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