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Secondary chemical equilibria in high-performance liquid chromatography: influence of ionic strength and pH on retention of peptides

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Abstract

In this work a model describing the effect of pH on retention in LC is established and tested for two series of peptides using an octadecylsilica column. The suggested model uses the pH value measured in the acetonitrile–water mixture 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 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: Dissociation constants; pH effects; Retention prediction; Mobile phase composition; Peptides

1. Introduction

The efficient isolation of peptides has become increasingly important for an ever-widening range of research disciplines in recent years. Advances in biotechnology have provided the ability to prepare peptides for therapeutic purposes. However, during biosynthesis, impurities very close to the desired peptide will be present and may require separation and purification [1–3]. LC has proved very versatile in separation and purification of peptides from a great variety of sources [1,3–5]. 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 peptide separation may be obtained by trial and error, this may take many attempts with subsequent loss of time and final peptide 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 peptides under different separation conditions.

The optimisation of chromatographic resolution of ionogenic solutes in LC is a task that has been actively researched [6–8]. Due to the specific acid–base characteristics of ionogenic solutes, the two most useful optimisation parameters are the pH and the organic modifier concentration.

In previous works [9–12] an approach for optimising concentration of organic modifier in the mobile phase has been tackled by establishing relationships between retention parameters and Reichardt's E_T^N scale of solvent polarity. Data from these linear

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relationships summarise an approach for optimising the organic modifier concentration from only two retention measurements for each compound [11]. Log k' values of peptides and E_T^N parameter values of the acetonitrile–aqueous phase eluent system correlate linearly, indicating that a good chromatographic separation can be obtained for the peptides studied when the percentage of acetonitrile in the mobile phase is 7% (v/v) for low mass peptides and 35% (v/v) for penta- and nonapeptides.

The inclusion of pH as an additional optimization parameter raises several problems [13]. The pH of the mobile phase is usually taken to be the same as that of the aqueous fraction. However, the pK values of the acids used to prepare the buffers change with the solvent composition [14,15] so does the pH of the buffer [16,17]. Sometimes the pH is measured after mixing the buffer with the organic modifier [18]. 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 acetonitrile–water mixtures [14].

pH measurements in acetonitrile–water, the most widely used mobile phase, can be preferred in a manner similar to that in water [19], taking into account the pH values previously assigned to primary standard buffer solutions in acetonitrile–water mixtures [10,16,17] according to the NIST multiprimary standard scale [20]. Also, in compliance with IUPAC rules [19–21], the activity coefficients of the species in acetonitrile–water mixtures can be calculated from the ionic strength through the classical Debye–Hückel equation [8].

In this work a model describing the effect of pH on retention in LC is established. The suggested model uses the pH value in the acetonitrile–water mixture used as mobile phase, instead of pH value in water and takes into account the effect of the activity coefficients. The model is tested for two series of peptides in two acetonitrile–water compositions respectively (7% and 35%, acetonitrile, v/v by volume). The usefulness of the proposed equations is twofold. They permit the determination of the acidity constants in the hydroorganic mobile phase, which are determined in this work from retention and pH measurements, and also permit the prediction of the pH optimum as a function of a minimum number of measurements. The proposed 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.

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 mS cm^{-1} and acetonitrile (Merck, Darmstadt, Germany) were HPLC grade. Trifluoroacetic acid (TFA), sodium hydroxide, potassium bromide and potassium hydrogen phthalate were all analytical grade obtained from Merck. All the peptides used in this study were purchased from Sigma (St. Louis, MO, USA). The low mass peptides (series A) are: L-2-amino-5-ureidovaleric acid (citrulline), *N*-(*N*-glycyl)-glycine (Gly–Gly), *N*-(*N*-glycylglycyl)-glycine (Gly–Gly–Gly), *N*-(*N*-γ-L-glutamyl-L-cisteinyl)-glycine (glutathione), *N*-(*N*-L-tirosylglycyl)-glycine (Tyr–Gly–Gly), *N*-(*N*-glycylglycyl)-L-valine (Gly–Gly–Val), *N*-(*N*-glycylglycyl)-L-isoleucine (Gly–Gly–Ile), *N*-(*N*-DL-alanyl-DL-leucyl)-glycine (Ala–Leu–Gly), *N*-(*N*-glycylglycyl)-L-phenylalanine (Gly–Gly–Phe). The penta, octa and nonapeptides are: octapeptide (Val–His–Leu–Thr–Pro–Val–Glu–Lys), lipressin (Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Lys–Gly–NH_2, disulfide bridge 1–6), oxytocin (Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2, disulfide bridge 1–6), Met-enkephalin (Tyr–Gly–Gly–Phe–Met), bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg) and Leu-enkephalin (Tyr–Gly–Gly–Phe–Leu). In the case of DL–Ala–DL–Leu–Gly, it was possible to separate two diastereoisomer mixtures. Citrulline, Gly–Gly and Gly–Gly–Gly were kept at room temperature and the remainder were stored in a freezer at 0°C when not in use. Stock solutions of the peptides were prepared by dissolving approximately 10 mg of each peptide and diluting to 5 ml; working solutions were prepared by

10-fold dilution of the stock solution. The mixtures of the peptides studied was prepared by 100-fold dilution of the stock solution. The solvent was a mixture acetonitrile–water (7:93), 0.05% (*v*/*v*) TFA for peptides of series A and a mixture acetonitrile–water (35:65), 0.1% (*v*/*v*) TFA for peptides of series B. 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. The chromatographic system was controlled by ChemResearch Chromatographic Data Management System Controller Software (ISCO) running on a Peceman AT Supermicro personal computer. A Merck LiChrospher 100 RP-18 (5 μm) column 250 × 4 mm I.D. was used at room temperature.

The 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 acetonitrile–water mixtures before the emf measurements, which were performed in triplicate to ensure potentiometric system stability.

2.3. Chromatographic procedure

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 6.5, with sodium hydroxide at two percentages of acetonitrile, 7% (series A) and 35% by volume (series B).

Capacity 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 peptides. The flow-rate of the mobile phase was maintained at 1 ml min^{-1}.

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_{PS}, for the standardization of potentiometric sensors in acetonitrile–water mixtures. This was assigned in previous works [16,17], in accordance with IUPAC rules [19,21,22] and on the basis of multiprimary standard scale, according to the National Institute of Standard and Technology (NIST) [20].

We use a standard reference solution, pH_{PS}, of potassium hydrogen phthalate and a commercial combination pH electrode, since good accuracy and precision were obtained for pH values up to 7, using this pH_{PS} with commercial electrode [10]. In addition, rapid stabilization of the potentiometric system was observed as shown in a previous work [10].

The molar activity coefficients, *y*, were calculated using the Debye–Hückel expression [23] taking into account that the ionic strength, *I*, of the mobile phases used can be easily calculated and remains constant over the entire range of pH explored.

3. Results and discussion

The capacity factor values, k', for the two series of peptides studied at different pH values of the mobile phase, were determined from three different injections at every mobile phase pH considered, and are shown in Figs. 1 and 2 and Fig. 3. Relative standard deviation lower than 2% for the k' values were obtained.

The peptides studied usually have two relevant functional groups, Fig. 4. pK values in the acid range can be associated with carboxylic acid function and pK values in the basic range can be assigned to the protonated amino groups dissociation [24]. Thus, peptides can be considered as typical zwitterion forming compounds. However, 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 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

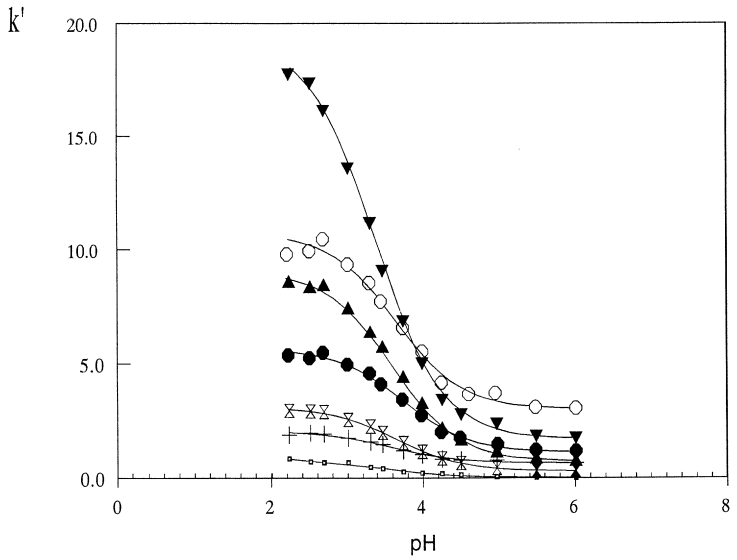

Fig. 1. Plots of the k' values of the low-molecular-mass peptides studied (series A) versus mobile phase pH with 7% (v/v) of acetonitrile. Glutathione (□), Tyr–Gly–Gly (+), Gly–Gly–Val (⧖), Ala–Leu–Gly I (●), Gly–Gly–Ile (▲), Ala–Leu–Gly (II) (○) and Gly–Gly–Phe (▼).

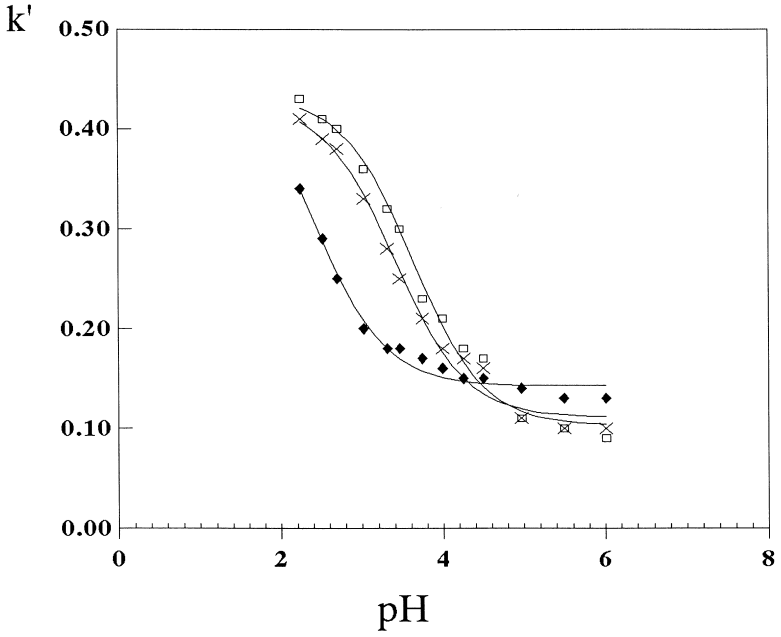

Fig. 2. Plots of the k' values of the low-molecular-mass peptides studied (series A) versus mobile phase pH with 7% (v/v) of acetonitrile. Citrulline (♦), Gly–Gly (×) and Gly–Gly–Gly (□).

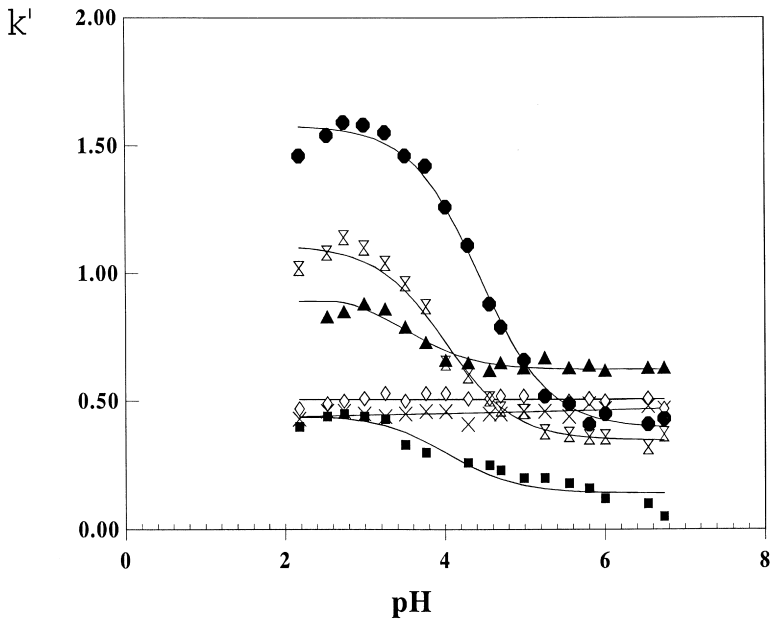

Fig. 3. Plots of the k' values of the penta, octa and nonapeptides studied (series B) versus mobile phase pH with 35% (v/v) acetonitrile. Octapeptide (■), lypressin (×), oxytocin (◇), bradykinin (▲), Met-enkephalin (⧖) and Leu-enkephalin (●).

view, with the widely used ODS stationary bonded phase, only the protolytic equilibria corresponding to pK values in the acid range are relevant. Glutathione and the denominated octapeptide present two pK values in the acid range corresponding to dissociation of two carboxylic groups. In the other cases only one pK value, corresponding to the dissociation of the terminal carboxylic group has been considered.

The retention of peptides is high at low pH values, Figs. 1–3, where the peptide exists as a single charged cation. When pH increases, the k' value decreases and lewels off at isoelectric point pH and stays constant. In the cases of gluthatione and octapeptide, as the pH is increased to pH values where the two carboxylic acids are dissociated, the k' values decreases again, Figs. 1 and 3. In the cases of oxytocin and lispressin no variation of chromatographic retention with pH was observed.

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 values, the pH, the 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 capacity factor as a function of pH of the mobile phase, considering the activity coefficient, and accounts for every peptide equilibria that influences the retention in ODS columns, can be derived taking into account that the ionization of peptides in the mobile phase takes place according to the following equilibrium:

$$H^+BAH \Leftrightarrow H^+BA^- + H^+ \qquad K_{a1}$$

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

Equations that relate the retention of a compound in LC columns with the pH of the eluent, consider that the observed capacity factor, k', is a weighted average of the k' of the ionic and neutral forms of the solute [25] according to the molar fractions of these forms in the mobile phase. The overall observed, k', for peptides can be given as:

$$k' = x_{H^+BAH} k'_{H^+BAH} + x_{H^+BA^-} k'_{H^+BA^-} \qquad (3)$$

where k'_{H+BAH} and k'_{H+BA-} are the capacity factors and x_{H+BAH} and x_{H+BA-} the molar fraction of the

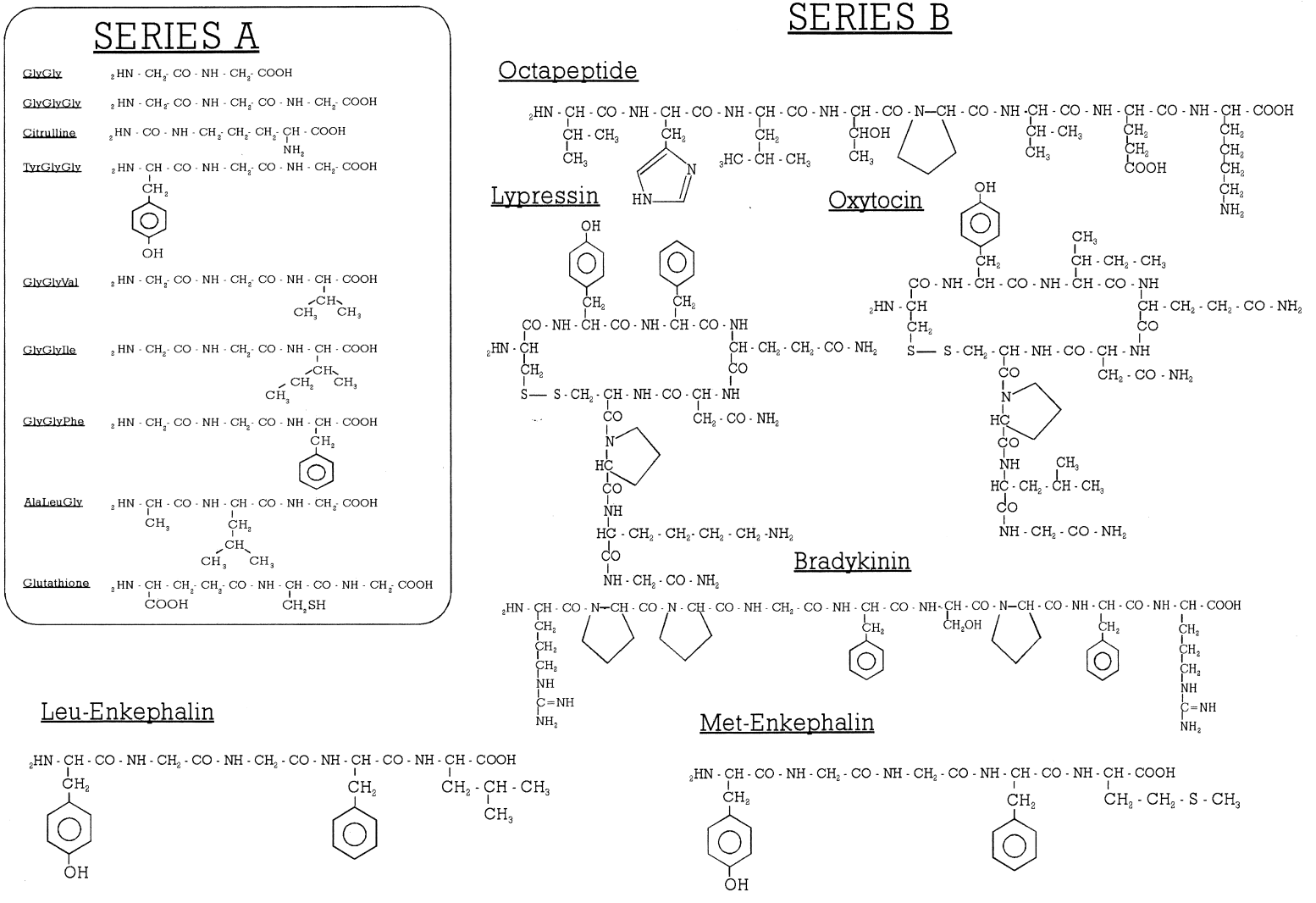

Fig. 4. Structures of peptides in study corresponding to series A and B.

protonated and zwitterionic forms of peptides respectively.

Eq. (3) can be written as

$$k' = \frac{[H^+BAH]k'_{H^+BAH} + [H^+BA^-]k'_{H^+BA^-}}{[H^+BAH] + [H^+BA^-]} \quad (4)$$

And, dividing by $[H^+BA^-]$

$$k' = \frac{\frac{[H^+BAH]}{[H^+BA^-]}k'_{H^+BAH} + k'_{H^+BA^-}}{\frac{[H^+BAH]}{[H^+BA^-]} + 1} \quad (5)$$

The protolytic equlibrium of peptides in the mobile phase is ruled by the thermodynamic dissociation constant,

$$K_{a1} = \frac{[H^+BA^-]a_{H^+}}{[H^+BAH]y} \quad (6)$$

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

From Eq. (6),

$$\frac{[H^+BAH]}{[H^+BA^-]} = \frac{a_{H^+}}{K_{a1}y} \quad (7)$$

By replacing Eq. (7) in Eq. (5):

$$k' = \frac{k'_{H^+BAH}\frac{a_{H^+}}{K_{a1}y} + k'_{H^+BA^-}}{\frac{a_{H^+}}{K_{a1}y} + 1} \quad (8)$$

In the special cases of glutathione, octapeptide and bradykinin different equilibria take place in the acid pH range. For glutathione, only the second equilibrium is considered because the first dissociation takes place at very low pH values and the capacity factors of acidic species cannot be determined:

$$H^+BAH_2^- \Leftrightarrow H^+BAH^{2-} + H^+ \qquad K_{a2}$$

For the octapeptide, the equilibria considered are:

$$H^{3+}BAH_2 \Leftrightarrow H^{3+}BAH^- + H^+ \qquad K_{a1}$$

$$H^{3+}BAH^- \Leftrightarrow H^{3+}BA^{2-} + H^+ \qquad K_{a2}$$

For the bradykinin:

$$H^{3+}BAH \Leftrightarrow H^{3+}BA^- + H^+ \qquad K_{a1}$$

The equations which relate the chromatographic retention and the pH of the mobile phase in the case of these three peptides can be derived in a similar way than Eq. (8) and are:

For glutathione:

$$k' = \frac{k'_{H^+BAH_2^-} + k'_{H^+BAH^{2-}}\frac{K_{a2}}{a_{H^+}y}}{\frac{K_{a2}}{a_{H^+}y} + 1} \quad (9)$$

For octapeptide:

$$k' = \frac{k_{H^{3+}BAH_2}\frac{a_{H^+}}{K_{a1}y^{3+}} + k'_{H^{3+}BAH^-} + k'_{H^{3+}BA^{2-}}\frac{K_{a2}y^{2+}}{a_{H^+}}}{\frac{a_{H^+}}{K_{a1}y^{3+}} + 1 + \frac{K_{a2}y^{2+}}{a_{H^+}}} \quad (10)$$

and for bradykinin:

$$k' = \frac{k_{H^{3+}BAH}\frac{a_{H^+}}{K_{a1}y^{3+}} + k_{H^{3+}BA^-}}{\frac{a_{H^+}}{K_{a1}y^{3+}} + 1} \quad (11)$$

The usefulness of Eqs. (8)–(11) is twofold. They can be effectively used to calculate the pK values of the substances and the capacity factors of the different species of the peptides, from the measured k' value and the pH and y variables. Secondly, these equations permit the prediction of the optimum eluting pH conditions from a minimum number of column k'-pH measurements. Each equation was experimentally verified and the pK values of the substances studied were determined from the experimental k' values, the pH measurements and calculated activity coefficient values. The obtained pK values are listed in Table 1 and were calculated by using a non-linear least-squares fit of the data to Eqs. (8)–(11). Table 1 also shows the capacity factors of the different species of the peptides. Figs. 1–3 show a good fit of the Eqs. (8)–(11) to experimental data. The curves

Table 1
Potentiometric (1) and chromatographic (2) pK_a values of peptides, and capacity factor values of species in acetonitrile–water mixtures

% Acetonitrile	7% (*v*/*v*)									
Method	Citrulline	Gly–Gly	Gly–Gly–Gly	Glutathione	Tyr–Gly–Gly	Gly–Gly–Val	Gly–Gly–Ile	Ala–Leu–Gly (I)	Ala–Leu–Gly (II)	Gly–Gly–Phe
	pK_{a1}	pK_{a1}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a1}	pK_{a1}	pK_{a1}	pK_{a1}	pK_{a1}
1	2.57 (0.03)	3.18 (0.01)	3.50 (0.02)	3.69 (0.01)	3.45 (0.01)	3.47 (0.02)	3.54 (0.03)	3.57 (0.02)	3.57 (0.02)	3.21 (0.02)
2	2.37 (0.11)	3.35 (0.06)	3.57 (0.06)	3.53 (0.05)	3.54 (0.05)	3.59 (0.02)	3.59 (0.02)	3.71 (0.04)	3.61 (0.05)	3.30 (0.02)
	k'_{H^+BAH}	k'_{H^+BAH}	k'_{H^+BAH}	$k'_{H^+BAH_2^-}$	k'_{H^+BAH}	k'_{H^+BAH}	k'_{H^+BAH}	k'_{H^+BAH}	k'_{H^+BAH}	k'_{H^+BAH}
	$k'_{H^+BA^-}$	$k'_{H^+BA^-}$	$k'_{H^+BA^-}$	$k'_{H^+BA^{2-}}$	$k'_{H^+BA^-}$	$k'_{H^+BA^-}$	k'_{H^+BA}	$k'_{H^+BA^-}$	$k'_{H^+BA^-}$	$k'_{H^+BA^-}$
	0.47 (0.04)	0.43 (0.01)	0.43 (0.01)	0.78 (0.02)	2.03 (0.04)	3.09 (0.02)	9.06 (0.08)	5.65 (0.08)	10.72 (0.19)	19.34 (0.21)
	0.14 (0.01)	0.11 (0.01)	0.10 (0.01)	0.02 (0.01)	0.65 (0.03)	0.29 (0.02)	0.72 (0.07)	1.11 (0.07)	2.99 (0.15)	1.70 (0.12)

% Acetonitrile	35% (*v*/*v*)				
Method	Bradykinin	Octapeptide		Met-enkephalin	Leu-enkephalin
	pK_{a1}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a1}
2	2.96 (0.14)	2.95 (0.14)	5.89 (0.29)	3.96 (0.06)	4.38 (0.04)
	$k'_{H^{3+}BAH}$	$k'_{H^{3+}BAH_2}$	$k'_{H^{3+}BA^{2-}}$	k'_{H^+BAH}	k'_{H^+BAH}
	$k'_{H^{3+}BA^-}$	$k'_{H^{3+}BAH}$		$k'_{H^+BA^-}$	k'_{H+BA^-}
	0.93 (0.03)	0.49 (0.02)	0.03 (0.04)	1.11 (0.02)	1.58 (0.02)
		0.62 (0.01)	0.21 (0.02)	0.35 (0.02)	0.39 (0.02)

Values in parentheses are standard deviations.

calculated using these equations are indicated as continuous lines and the plotted points are experimental data.

The advantages of the determination of the pK_a values using this chromatographic method over others are that only 1 mg or less of the substance is needed for the acidity constant determination 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 pK_a values were confirmed using the potentiometric method with the same solvent composition used in the LC method [26]. The greater pK_{a1} values of di and tripeptides than the corresponding pK_{a1} of monomeric amino acid, can be explained by the fact that in monomeric amino acids the carboxylic and amino groups are adjacent [27]. In effect, pK_{a1} values corresponding to the C-terminal carboxylic group obtained are approximately 1 pK_a unit greater than the relevant values of the given monomeric amino acid showing the same order (Gly, pK_1 = 2.35; Tyr, − = 2.20; Val, pK_{a1} = 2.24; Ile, pK_{a1} = 2.33 and Phe, pK_{a1} = 2.58) [28]. For citrulline, the obtained pK_{a1} is similar to the pK_{a1} value of other amino acids, since in this substance the carboxylic and amino groups are adjacent, while the amide group does not ionize in any way in the pH interval studied [29].

It is seen that the chromatographic and potentiometric pK values, listed in Table 1, are in good agreement and randomly deviate from each other about 5%.

On the other hand, Eqs. (8)–(11) can be written in a linearized form:

$$k'\left(\frac{\boldsymbol{a}_{\mathbf{H}+}}{\boldsymbol{K}_{\mathbf{a1}}\boldsymbol{y}}+\mathbf{1}\right)=k'_{\mathrm{H^+BAH}}\frac{\boldsymbol{a}_{\mathbf{H}^+}}{\boldsymbol{K}_{\mathbf{a1}}\boldsymbol{y}}+k'_{\mathrm{H^+BA^-}} \quad (12)$$

For glutathione,

$$k'\left(\frac{\boldsymbol{a}_{\mathbf{H}+}\boldsymbol{y}}{\boldsymbol{K}_{\mathbf{a2}}}+\mathbf{1}\right)=k'_{\mathrm{H^+BAH_2^-}}\frac{\boldsymbol{a}_{\mathbf{H}^+}\boldsymbol{y}}{\boldsymbol{K}_{\mathbf{a2}}}+k'_{\mathrm{H^+BAH^{2-}}} \quad (13)$$

For octapeptide,

$$k'\left(\frac{\boldsymbol{a}_{\mathbf{H}+}}{\boldsymbol{K}_{\mathbf{a2}}\boldsymbol{y}}+\mathbf{1}\right)=k'_{\mathrm{H^{3+}BAH_2}}\frac{\boldsymbol{a}_{\mathbf{H}^+}}{\boldsymbol{K}_{\mathbf{a1}}\boldsymbol{y}^{\mathbf{3+}}}+k'_{\mathrm{H^{3+}BAH^-}} \quad (14)$$

For bradykinin,

$$k'\left(\frac{\boldsymbol{a}_{\mathbf{H}+}}{\boldsymbol{K}_{\mathbf{a1}}\boldsymbol{y}^{\mathbf{3+}}}+\mathbf{1}\right)=k'_{\mathrm{H^{3+}BAH}}\frac{\boldsymbol{a}_{\mathbf{H}^+}}{\boldsymbol{K}_{\mathbf{a1}}\boldsymbol{y}^{\mathbf{3+}}}+k_{\mathrm{H^{3+}BA^-}} \quad (15)$$

When pK values of substance are known, plots of the bold terms can be used in order to optimize the pH of the mobile phase. Figs. 5 and 6 show these plots for the peptides studied. From these it is also possible to obtain k' values of the species of the peptides from the intercept and slope.

Having verified the linearity of Eq. (12) Eq. (13), Eq. (14) and Eq. (15), it should be very useful in practice taking into account the large number of pK_a values that are known in acetonitrile–water mixtures [30]. Also, estimation of pK values in acetonitrile–water mixture can be possible, if pK values in water are known, taking into account the preferential solvation by water in these mixtures [31]. Eq. (12) Eq. (13), Eq. (14) and Eq. (15) can be applied to other compounds, whose pK values are known, and only two experimental values at two different pH would be needed for the optimization of the pH of the mobile phase.

In order to examine the accuracy of retention predictions using Eq. (12) Eq. (13), Eq. (14) and Eq. (15), from just two measurements of k' for each compound, only data measured at pH 2.5 and pH 6.0 in acetonitrile–water mixtures as mobile phase were considered. From only two measurements by compound, k' values of peptides at different pH values were calculated, Figs. 5 and 6, taking into account the pK_a values of the peptides. Thus, selectivity was obtained for solute adjacent pairs in the usual way $\alpha = k'_2/k'_1$. In Figs. 7 and 8 the solid lines indicate α values obtained from two measurements by compound using Eqs. (12)–(15). The points shown are α values obtained from experimental data. Figs. 7 and 8 show that virtually the same values of α are obtained from the two methods. Thus it appears clear that two measurements of k' by compound are sufficient to predict accurately the optimum pH of the mobile phase if pK values are known.

Fig. 7 indicates that good chromatographic separation can be obtained for the peptides considered in series A with acetonitrile–water mixtures with 7% (v/v) of acetonitrile [9] at a pH of the hydro-organic mixtures between 2.5 and 3. Fig. 9 shows a chro-

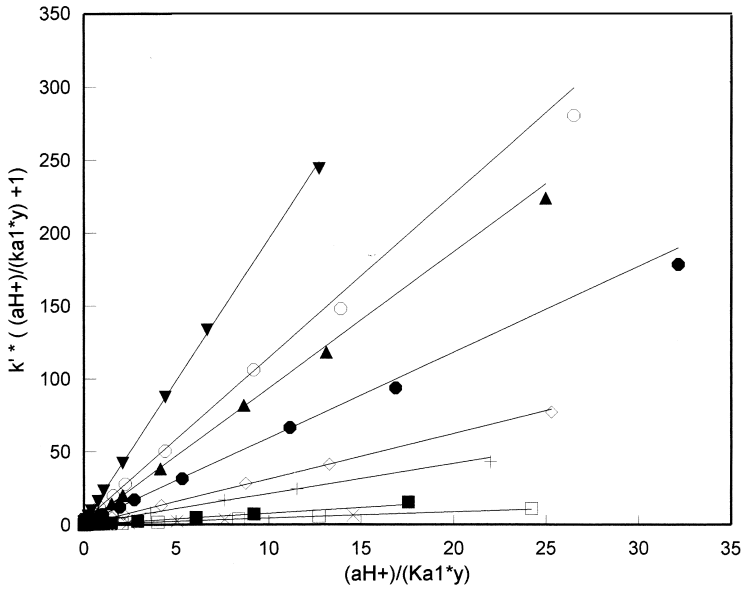

Fig. 5. Plots of $k'(1 + a_{H^+}/K_a y)$ versus $a_{H^+}/K_a y$ of the peptides of series A. Gly–Gly–Val (◇) and the remainder of the symbols as Fig. 1 and Fig. 2.

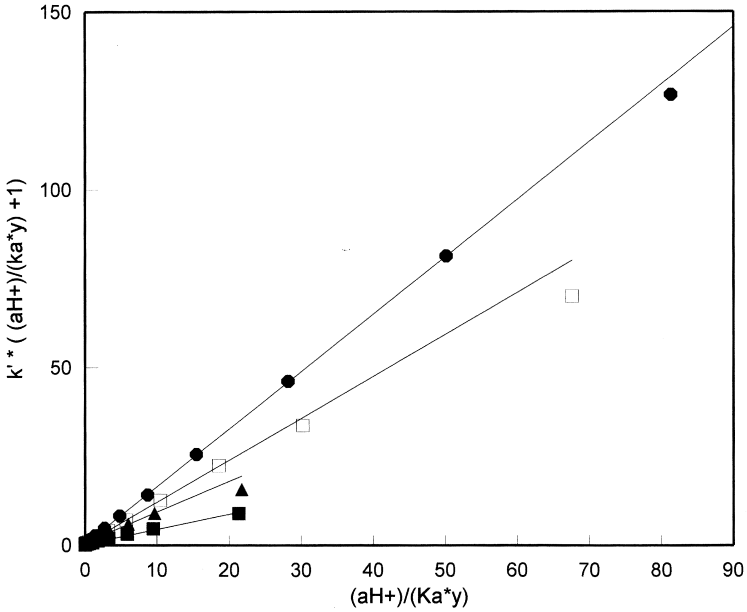

Fig. 6. Plots of $k'(1 + a_{H^+}/K_a y)$ versus $a_{H^+}/K_a y$ of the peptides of series B. Met-enkephalin (□) and the remainder of symbols as Fig. 3.

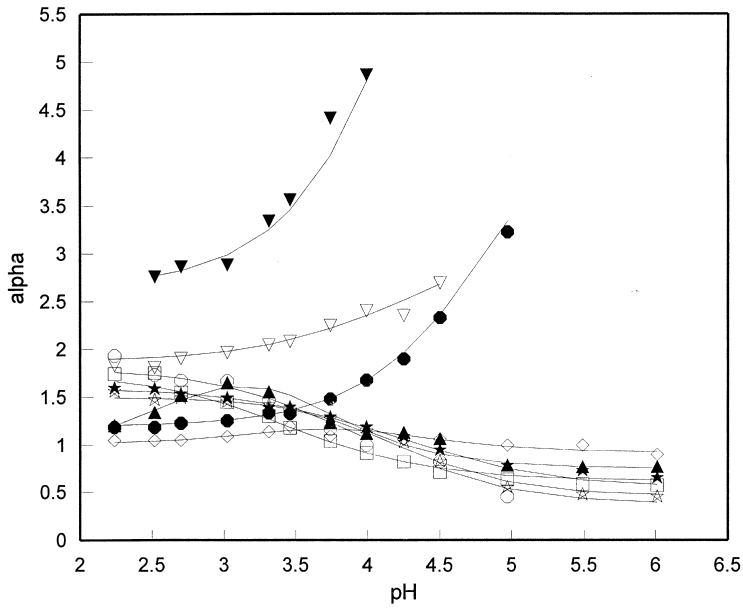

Fig. 7. Plots of α versus pH of peptides of series A. Gly–Gly/citrulline (▲), Gly–Gly–Gly/Gly–Gly (◇), glutathione/Gly–Gly–Gly (○), Tyr–Gly–Gly/glutathione (▼), Gly–Gly–Val/Tyr–Gly–Gly (☆), Ala–Leu–Gly I/Gly–Gly–Val (∇), Gly–Gly–Ile/Ala–Ala–Leu–Gly (I) (★), Ala–Leu–Gly (II)/Ala–Leu–Gly (I) (●) and Gly–Gly–Phe/Ala–Leu–Gly (II) (□).

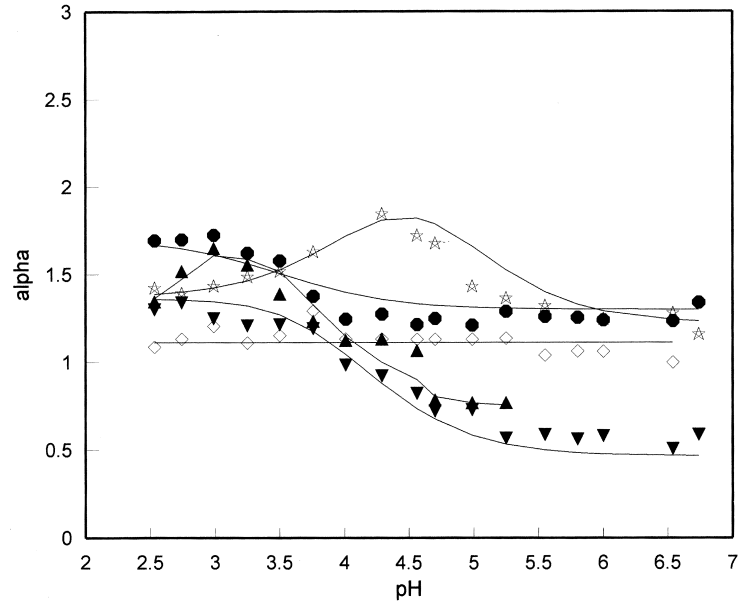

Fig. 8. Plots of α versus pH of peptides of series B. Lypressin/octapeptide (▲), oxytocin/lypressin (◇), bradykinin/oxitocin (●), Met-enkephalin/bradykinin (▼) and Leu-enkephalin/Met-enkephalin (☆).

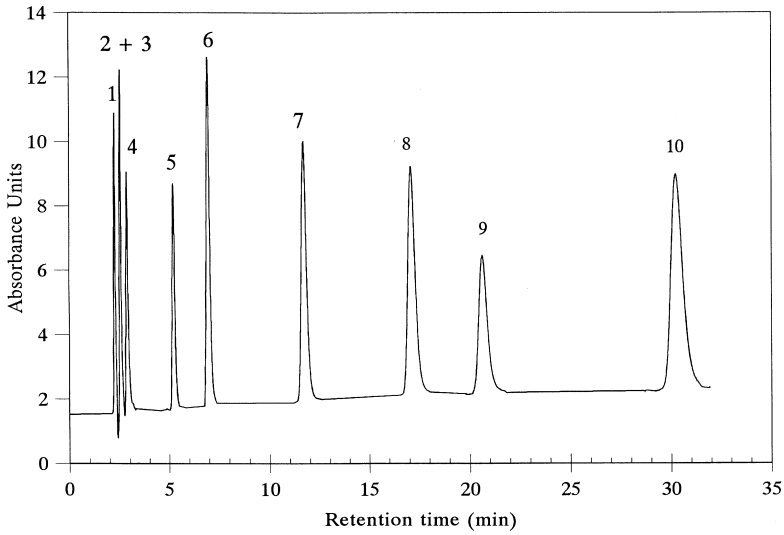

Fig. 9. Separation of citrulline (1), Gly–Gly+Gly–Gly–Gly (2+3), glutathione (4), Tyr–Gly–Gly (5), Gly–Gly–Val (6), Ala–Leu–Gly (I) (7), Gly–Gly–Ile (8), Ala–Leu–Gly (II) (9), Gly–Gly–Phe (10) with a mobile phase consisting on acetonitrile–water (7:93, *v*/*v*), 0.05% (*v*/*v*) trifluoroacetic acid adjusted the mobile phase pH to 2.8 with sodium hydroxide.

matogram of the separation of the ten substances studied in series A at pH=2.8. In a similar way, Fig. 8 indicates that the best separation for the peptides of series B was achieved with acetonitrile–water mixtures containing a percentage of acetonitrile of 35% (*v*/*v*), at a pH values between 3 and 3.5. Fig. 10 shows the chromatogram of the separation of the six peptides studied in series B at pH=3.3.

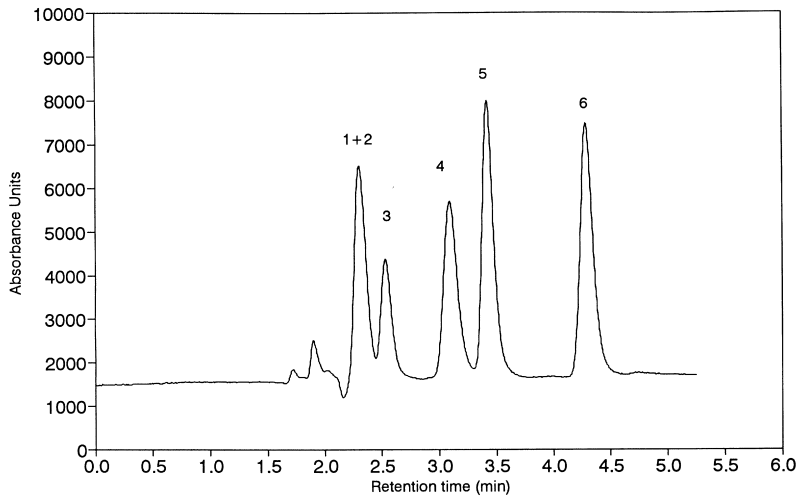

Fig. 10. Separation of octapeptide+lyspressin (1+2), oxytocin (3), bradykinin (4), Met-enkephalin (5) and Leu-enkephalin (6) with a mobile phase consisting on acetonitrile–water (35:65, *v*/*v*), 0.1% (*v*/*v*) trifluoroacetic acid adjusted the mobile phase pH to 3.3 with sodium hydroxide.

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