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Separation of potentially therapeutic peptide hormones by liquid chromatography
Optimisation of the composition and pH of the mobile phase

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Abstract

The aim of this work is to optimise the proportion of the organic modifier and the pH of the mobile phase, in order to separate a series of peptide hormones with therapeutic interest in the molecular mass range from 500 to 6000. The composition of the mobile phase was optimised by establishing relationships between retention parameters and either the scale of solvent polarity, or the Kamlet–Taft multiparameter solvent scale of the eluent, using linear solvation energy relationships. Likewise, linear correlations between the chromatographic retention and Reichardt's E_T^N parameter were obtained. These relationships allowed an important reduction of the experimental retention data needed for developing a given separation. In addition, a model describing the effect of the correctly measured pH of the mobile phase on retention in LC was established and tested for the series of selected peptides using an octadecylsilica column. The proposed equations permit the prediction of the optimum pH and also permit the determination of the acidity constants of the peptides in the hydro-organic mixtures using a minimum number of measurements.

Keywords: Mobile phase optimisation; Linear solvation energy relationships; Dissociation constants; Peptides; Hormones

1. Introduction

An increasing number of peptides are available for biomedical research and therapeutical applications. Once the structure and chemical properties of an active compound are known, quantitative and qualitative analytical methods are employed to detect and quantify it. Liquid chromatography (LC) has been the favourite and the most often used analytical tool for the analysis of complex peptide mixtures [1–3].

The retention behaviour of a peptide is the result of a complex interaction with both stationary and mobile phases. Although the stationary phase has an active role in the separation process, most researchers have focused attention on mobile phase optimisation, since this is the easiest way to control retention and selectivity in LC [4]. Due to specific acid–base characteristics of ionogenic solutes, the two most useful optimisation parameters are the organic modifier concentration and the pH of the mobile phase [5–7]. In order to avoid long empirical optimisation procedures, a system for predicting peptide retention times is of great advantage. In this way, linear solvation energy relationships (LSERs) formalism, has been used successfully to study retention in LC [8–11] and seeks to relate chromatographic retention to variations in characteristic prop-

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erties of mobile phases such as the solvatochromic parameter π^*, that evaluates solvent dipolarity/polarisability [12] and parameters α, β, that evaluate solvent hydrogen-bond acidity [13] and solvent hydrogen-bond basicity [14], respectively. The general equation is:

$$\log k = (\log k)_0 + M(\delta_s^2 - \delta_m^2)\overline{V}_2/100 + S(\pi_s^* - \pi_m^*)\pi_2^* + A(\alpha_s - \alpha_m)\beta_2 + B(\beta_s - \beta_m)\alpha_2 \quad (1)$$

Here, k is the chromatographic capacity factor; $(\log k)_0$ is the intercept of the regression equation, $\overline{V}_2$ is the molar volume of the solute, δ^2 is the square of the Hildebrand solubility parameter (a measure of the solvent cohesive energy) and π^*, α and β are the Kamlet–Taft solvatochromic parameters. Subscripts s and m denote the stationary and mobile phases, respectively and subscript 2 refers to the solute properties. The values M, S, A and B are the correlation coefficients for this equation and do not depend on the solute [15]. The solute–solute interaction term proposed by Lee [16] was not considered in this work. When a system with a fixed pair of solute and stationary phase is considered, and assuming the invariance of the properties of the stationary phase with the change in the mobile phase composition [15] and the relationship between δ^2 and the solvatochromic parameters of the mobile phase [15,17], Eq. (1) can be simplified to:

$$\log k = (\log k)_0 + s\pi_m^* + a\alpha_m + b\beta_m \quad (2)$$

where $(\log k)_0$ depends on the parameters of the stationary phase and the solute s, a and b are the correlation coefficients which depend on the solute parameters, and π_m, α_m and β_m are the Kamlet–Taft solvatochromic parameters of the mobile phase. Eq. (2) allows the chromatographic behaviour of the studied solutes to be predicted for any composition of the eluent system and it has been widely verified with different substances [3,11,18]. Taking into account that β values of acetonitrile are nearly constant over most of the composition range [19,20], and the observed correlation between the normalised Dimroth and Reichardt polarity parameter, E_T^N [21], and π^* and α solvatochromic parameters, $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [20], Eq. (2) can be reduced to a single solvent parameter dependent expression:

$$\log k = C + eE_T^N \quad (3)$$

Eq. (3) has been widely used to correlate chromatographic retention of different solutes with the polarity of the mobile phases [1,3,11,18,22,23], and the good linearity obtained indicates that suitable prediction for a specific solute in a fixed stationary phase can be achieved from the E_T^N of the eluent and a few experimental data.

These approaches only allow the prediction of retention at different mobile phase compositions, but do not provide information about the pH of the mobile phase, which is important in understanding the retention process. The pH of the mobile phase is also critical for optimising selectivity in LC since the degree of ionisation of solutes, stationary phases and mobile phase additives may be affected by the pH [5,24]. The pH measurements in a mixed solvent can be performed as easily as in water taking into account the operational definition of pH [25–27]:

$$pH_X = pH_S + \frac{E_S - E_X}{g} \quad (4)$$

where E_X and E_S denote the electromotive force (e.m.f.) measurements on the sample solution at unknown pH_X and on the standard primary reference solution at known pH_S, respectively, and $g = (\ln 10)RT/F$.

In previous works, a model describing the effect of pH on retention in LC was established [28,29]. In this work, equations, which relate the capacity factor of the peptide hormone studied and the pH of the mobile phase, were developed, taking into account the ionization equilibria of the studied peptides. The suggested model uses the pH value in acetonitrile–water mixture used as mobile phase, instead of pH value in water, and considers the effect of the activity coefficients.

In this work, the proportion of the organic modifier and the pH of the hydro-organic mobile phase were optimised in order to separate a series of 10 peptides with molecular masses ranging from 500 to 6000. This series of peptide hormones was chosen so

as to cover a wide molecular mass range of peptides of high therapeutical and pharmacological interest. The LSER method, based either on the multiparameter π^*, α and β scale or the single solvent parameter E_T^N, and the relationships with log k, have been applied to the optimisation of the mobile phase composition and to the prediction of the chromatographic behaviour of peptide hormone substances. Moreover, the pH measurements in the acetonitrile–water mixtures used as mobile phases and their correlation with k have been used in the optimisation of the mobile phase pH for the required separation. Likewise, this relationship between retention and mobile phase pH also made it possible to determine the dissociation constants studied in acetonitrile–water of the peptide hormones from the same retention measurements already used for the optimisation of the mobile phase pH. The usefulness of the proposed equations is twofold. First, they permit the prediction of the optimum pH as a function of a minimum number of measurements and secondly, permit the determination of the acidity constants in the hydro–organic mobile phase using the same experimental data.

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, potassium bromide and potassium hydrogenphthalate (dried at 110°C before use) were all analytical grade obtained from Merck. The peptides used in this study: Val–His–Leu–Thr–Pro–Val–Glu–Lys (VHLTPVEK), lypressin, oxytocin, bradykinin, Met-enkephalin, Leu-enkephalin and bovine insulin were purchased from Sigma (Alcobendas, Madrid, Spain), buserelin from Hoechst Ibérica (Barcelona, Spain), triptorelin from Lasa (Barcelona, Spain) and salmon calcitonin from Lipotec (Barcelona, Spain). The names of the peptides with their amino acid sequence and molecular mass are shown in Table 1. All the amino acids except glycine and several of those in the sequence of buserelin and triptorelin have L-configuration. All the peptides were stored in a freezer at 0°C when not in use. Stock solutions of the peptides were prepared by

Table 1
International name, amino acid sequence and molecular mass (M_r) of peptides studied

Peptide	Amino acid sequence	M_r
N-(*N*-L-Valyl-L-histidyl-D-leucyl-L-threonyl-L-prolyl-L-valyl-L-glutamyl)-L-lysine (VHLTPVEK)[a]	Val–His–Leu–Thr–Pro–Val–Glu–Lys	922.1
Lypressin	Cys–Tyr–Phe–Gln–Asn–Cys–Pro–Lys–Gly–NH_2 cyclic (1–6) disulphide	1056.2
Oxytocin	Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2 cyclic (1–6) disulphide	1007.2
Bradykinin	Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg	1060.2
Met-Enkephalin	Tyr–Gly–Gly–Phe–Met	573.1
Leu-Enkephalin	Tyr–Gly–Gly–Phe–Leu	555.6
Triptorelin	pGlu–Pro–His–Trp–Ser–Tyr–D-Trp–Leu–Arg–Pro–Gly–NH_2	1311.5
Buserelin	pGlu–Pro–His–Trp–Ser–Tyr–D-Ser(*tert*.-Bu)–Leu–Arg–Pro–NHC_2H_5	1239.4
Bovine insulin	Chain A: Gly–Ile–Val–Glu–Gln–Cys–Cys–Ala–Ser–Val–Cys–Ser–Leu–Tyr–Gln–Leu–Glu–Asn–Tyr–Cys–Asn Chain B: Phe–Val–Asn–Gln–His–Leu–Cys–Gly–Ser–His–Leu–Val–Glu–Ala–Leu–Tyr–Leu–Val–Cys–Gly–Glu–Arg–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala cyclic (A7–B7, A20–B19, A6–A11) disulphide	5733.5
Salmon calcitonin	Cys–Ser–Asn–Leu–Ser–Thr–Sys–Val–Leu–Gly–Lys–Leu–Ser–Gln–Glu–Leu–His–Lys–Leu–Gln–Thr–Tyr–Pro–Arg–Thr–Asn–Thr–Gly–Ser–Gly–Thr–Pro–NH_2 cyclic (1–7) disulphide	3431.9

[a] Abbreviated by the one-letter code of the constituent amino acid residues.

dissolving approximately 5 mg of each peptide and diluting to 5 ml; working solutions were prepared by 10-fold dilution of the stock solution. The mixture of the 10 peptides studied was prepared by 100-fold dilution of the stock solution. The hydro-organic mixtures used as solvents were acetonitrile–water, 0.1% (v/v) TFA. All the eluents and mobile phases were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by sonication, and 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 Sotfware (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.

The e.m.f. 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 externally thermostated at 25±0.1°C. The electrodes were stabilised in the appropriate acetonitrile–water mixtures before the e.m.f. measurements, which were performed in triplicate to ensure potentiometric system stability.

2.3. Chromatographic procedure

For the optimisation of mobile phase composition, the solution was made of different acetonitrile–water mixtures containing 0.1% (v/v) trifluoroacetic acid [30–33], with the pH of the mobile phase adjusted to 2.2 with sodium hydroxide at several acetonitrile percentages, up to 60% (v/v). The LiChrospher C_{18} column was equilibrated with new mobile phase conditions for 30 min. The flow-rate of the mobile phase was 1 ml/min and the signal was monitored at 214 nm. Capacity factors were calculated from $k=(t_R-t_0)/t_0$, where t_0 was the hold-up time, and t_R the retention time of each peptide for every mobile phase. The hold-up time, t_0, was established for every mobile phase composition using potassium bromide solution [0.01% (w/v) in water] [34]. The retention times and the capacity factors of the solutes were determined from three different injections at every mobile phase composition considered.

In order to study the influence of the eluent pH on the chromatographic separation, the mobile phase was adjusted to different pH values, from 2.2 to 6.5, with sodium hydroxide. 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 of the National Institute of Standard and Technology (NIST) [26], pH_S, for the standardisation of potentiometric sensors in acetonitrile–water mixtures. This was assigned in previous works [35–37], in accordance with IUPAC rules [27]. A standard reference solution of potassium hydrogenphthalate and a commercial combination pH electrode were used owing to the good accuracy and precision obtained for pH values up to 7 [11].

2.4. Data analysis

Optimisation of the mobile phase composition was carried out by means of the LSER approach, using the multiparametric relationships between the capacity factor of the peptides and the solvatochromic parameters of the mobile phases studied in accordance with Eqs. (2) and (3).

To optimise the pH of the mobile phase and to calculate the pK values of the studied peptides, theoretical models describing the dependence of the capacity factors, k, on the pH of the mobile phase were derived taking into account pH values and activity coefficients in the mobile phase. The ionization equilibria of the compounds and the equations are:

For Met-enkephalin and Leu-enkephalin,

$$H^+BAH_2 \Leftrightarrow H^+BAH^- + H^+ \quad K_{a1} \tag{5}$$

$$k = \frac{k_{+1}\dfrac{a_{H^+}}{K_{a1}y_i} + k_0}{\dfrac{a_{H^+}}{K_{a1}y_i} + 1} \tag{6}$$

For bradykinin,

$$H^{3+}BAH \Leftrightarrow H^{3+}BA^{-} + H^{+} \quad K_{a1} \tag{7}$$

and for triptorelin and buserelin,

$$H^{3+}BAH \Leftrightarrow H^{2+}BAH + H^{+} \quad K_{a1} \tag{8}$$

$$k = \frac{k_{+3}\dfrac{a_{H^+}y_i^2}{K_{a1}y_i^3} + k_{+2}}{\dfrac{a_{H^+}y_i^2}{K_{a1}y_i^3} + 1} \tag{9}$$

For the octapeptide, Val–His–Leu–Thr–Pro–Val–Glu–Lys,

C-terminal carboxylic

$$H^{3+}BAH_2 \Leftrightarrow H^{3+}BAH^{-} + H^{+} \quad K_{a1} \tag{10}$$

Carboxylic glutamic

$$H^{3+}BAH^{-} \Leftrightarrow H^{3+}BA^{2-} + H^{+} \quad K_{a2} \tag{11}$$

Histidine

$$H^{3+}BAH^{2-} \Leftrightarrow H^{2+}BA^{2-} + H^{+} \quad K_{a3} \tag{12}$$

$$k = \frac{k_{+3}\dfrac{(a_{H^+})^3}{K_{a1}K_{a2}K_{a3}y_i^3} + k_{+2}\dfrac{(a_{H^+})^2}{K_{a2}K_{a3}y_i^2} + k_{+1}\dfrac{a_{H^+}}{K_{a3}y_i} + k_0}{\dfrac{(a_{H^+})^3}{K_{a1}K_{a2}K_{a3}y_i^3} + \dfrac{(a_{H^+})^2}{K_{a2}K_{a3}y_i^2} + \dfrac{a_{H^+}}{K_{a3}y_i} + 1} \tag{13}$$

3. Results and discussion

The logarithm of the capacity factor values (log k) were obtained at different percentages of acetonitrile for the peptides studied (Table 2). Acetonitrile–water mixtures containing 0.1% (v/v) TFA in (15:85), (20:80), (22.5:72.5), (25:75), (30:70), (32:68), (35:65), (40:60), (45:55), (50:50) and (60:40) ratios were assayed as mobile phases. Chromatographic retention has been correlated with properties of the hydroorganic mixtures used as mobile phases, that is the Kamlet–Taft solvatochromic parameters π^*, α and β [12–14], in order to evaluate the effect of the solute–solvent interactions on the capacity factors. Values of π^*, α, β [20,38], together with E_T^N values [39] for all the acetonitrile–water mixtures used, are known for the whole range of compositions and are given in Table 3.

The coefficients s, a and b of the correlation defined by Eq. (2) were obtained by multiple linear regression of log k and these above mentioned mobile phase properties. Table 4 shows the multiparametric relationships between the capacity factors of the peptides and the solvatochromic parameters of the acetonitrile–water mixtures used as mobile phases, with percentages of acetonitrile from 15 to 60% (v/v).

Log k correlates well with the solvatochromic parameters π^*, α and β, since the average coefficient value (r) was higher than 0.99 using multiple

Table 2
Values of the logarithm of the capacity factors of the peptides studied at various percentages of acetonitrile (ACN) in the mobile phase[a]

ACN (%)	Log k									
	VHLTPVEK	Lypressin	Oxytocin	Bradykinin	Met-Enkephalin	Leu-Enkephalin	Triptorelin	Buserelin	Insulin	Calcitonin
60	−0.594 (0.001)	−0.638 (0.021)	−0.712 (0.028)	−0.755 (0.001)	−0.510 (0.016)	−0.454 (0.004)	−0.673 (0.006)	−0.536 (0.004)	−1.104 (0.016)	−0.939 (0.028)
50	−0.485 (0.003)	−0.638 (0.001)	−0.626 (0.009)	−0.493 (0.004)	−0.366 (0.001)	−0.240 (0.011)	−0.411 (0.002)	−0.298 (0.003)	−0.584 (0.004)	−0.615 (0.008)
45	−0.480 (0.001)	−0.597 (0.001)	−0.567 (0.001)	−0.435 (0.003)	−0.266 (0.002)	−0.159 (0.003)	−0.238 (0.002)	−0.123 (0.002)	−0.504 (0.001)	−0.301 (0.001)
40	−0.437 (0.003)	−0.430 (0.001)	−0.403 (0.003)	−0.331 (0.006)	−0.166 (0.001)	−0.016 (0.001)	−0.044 (0.007)	0.070 (0.001)	−0.227 (0.005)	0.223 (0.001)
38	−0.443 (0.003)	−0.395 (0.001)	−0.365 (0.001)	−0.237 (0.003)	−0.088 (0.001)	0.060 (0.005)	0.071 (0.002)	0.192 (0.003)	−0.024 (0.010)	0.543 (0.003)
35	−0.400 (0.001)	−0.364 (0.001)	−0.330 (0.003)	−0.154 (0.011)	0.008 (0.001)	0.172 (0.003)	0.282 (0.002)	0.413 (0.003)	0.352 (0.001)	1.065 (0.003)
32	−0.340 (0.003)	−0.324 (0.001)	−0.234 (0.002)	0.000 (0.002)	0.124 (0.001)	0.306 (0.002)	0.525 (0.002)	0.667 (0.001)	0.915 (0.005)	1.693 (0.003)
30	−0.329 (0.004)	−0.289 (0.007)	−0.126 (0.003)	0.117 (0.003)	0.213 (0.001)	0.411 (0.001)	0.712 (0.001)	0.863 (0.002)	1.406 (0.011)	–
28	−0.301 (0.002)	−0.212 (0.004)	−0.010 (0.002)	0.266 (0.001)	0.326 (0.003)	0.545 (0.002)	0.945 (0.001)	1.103 (0.001)	–	–
25	−0.168 (0.002)	−0.010 (0.001)	0.228 (0.001)	0.482 (0.002)	0.472 (0.002)	0.739 (0.002)	1.304 (0.001)	1.445 (0.006)	–	–
22.5	0.021 (0.001)	0.198 (0.001)	0.510 (0.001)	0.797 (0.001)	0.687 (0.001)	1.965 (0.001)	1.713 (0.001)	1.875 (0.001)	–	–
20	0.239 (0.001)	0.392 (0.004)	0.788 (0.001)	1.149 (0.003)	0.905 (0.001)	1.029 (0.001)	–	–	–	–
15	0.869 (0.003)	1.041 (0.004)	1.380 (0.002)	–	1.448 (0.007)	1.811 (0.002)	–	–	–	–

[a] Values in parentheses are standard deviation.

Table 3
Solvatochromic parameters values for the acetonitrile–water mixtures studied

ACN (%, v/v)	E_T^N	π^*	α	β
60	0.78	0.91	0.91	0.60
50	0.80	0.95	0.91	0.61
45	0.81	0.97	0.91	0.61
40	0.83	1.00	0.91	0.61
38	0.83	1.00	0.91	0.61
35	0.84	1.02	0.92	0.61
32	0.85	1.03	0.93	0.61
30	0.86	1.04	0.94	0.61
28	0.87	1.05	0.95	0.61
25	0.88	1.06	0.96	0.61
22.5	0.89	1.07	0.97	0.61
20	0.90	1.08	0.98	0.61
15	0.92	1.10	1.01	0.60

linear regression for the data sets examined here. The sign of the magnitude of the coefficients *s*, *a*, and *b* measures the direction and relative strength of different types of solute–mobile phase interactions affecting retention for a given compound in a fixed stationary phase. The positive sign for the coefficient *s* indicates that increasing mobile phase dipolarity (π^*) leads to an increase in the retention. The sign of the coefficient *b* is negative for the most of the peptides, which indicates that increasing the mobile phase hydrogen bond basicity (β) leads to a decrease in the solute retention. The positive sign of the coefficient *a* indicates that an increase in the hydrogen bond acidity (α) of the mobile phase leads to an increase in the chromatographic retention. When the proportion of water in the mobile phase is increased the dipolarity of the mobile phase is also increased, the hydrogen bond basicity values are slightly decreased and the hydrogen bond acidity are increased (Table 3), therefore the solutes are retained longer on the apolar stationary phase. These conclusions agree with the general theory in reversed-phase liquid chromatography indicating that retention is greater when proportion of water in the mobile phase increases.

Eq. (2) can be used to describe and predict the chromatographic retention of the studied peptides from the dipolarity and hydrogen bond properties of the acetonitrile–water mixture used as mobile phase. In order to examine the accuracy of retention predictions using Eq. (2), log *k* values predicted with the LSER equations versus experimental log *k* values are plotted in Fig. 1. A good accuracy of retention predictions was obtained for every peptide.

The experimental log *k* values were also related with the E_T^N solvent parameter according to Eq. (3), as shown in Fig. 2. All the peptides showed a similar elution profile. Log *k* and E_T^N correlate linearly over the whole experimental range of acetonitrile contents studied, but there are two straight lines with different slopes, which intersect at acetonitrile percentages of roughly 30% (v/v).

These two straight lines could be explained taking into account the structural features of acetonitrile–water mixtures, described by Marcus and Migron [20], which show three regions. On the water-rich side there is a region in which the structure of the water molecules remains more or less intact, the acetonitrile molecules gradually occupy the cavities between them with little disruption of the water structure. The limit of acetonitrile molar fraction

Table 4
Relationships between log *k* for the peptides studied and π^* α, and β solvatochromic parameters of the eluent system in the interval studied using the LSER approach

Substance	Multiparametric relationships	*r*
VHLTPVEK	$\log k = +18.02 - 1.43\pi^* + 10.97\alpha - 44.44\beta$	0.990
Lypressin	$\log k = +13.04 + 1.03\pi^* + 10.79\alpha - 40.06\beta$	0.990
Oxytocin	$\log k = -12.30 + 1.77\pi^* + 16.06\alpha - 7.60\beta$	0.991
Bradykinin	$\log k = -32.50 + 1.43\pi^* + 18.59\alpha + 22.55\beta$	0.992
Met-Enkephalin	$\log k = -6.22 + 4.17\pi^* + 10.36\alpha - 12.43\beta$	0.995
Leu-Enkephalin	$\log k = -9.80 + 4.67\pi^* + 12.31\alpha - 10.05\beta$	0.996
Triptorelin	$\log k = -25.56 + 6.74\pi^* + 20.30\alpha + 0.46\beta$	0.997
Buserelin	$\log k = -23.23 + 7.10\pi^* + 20.45\alpha - 3.97\beta$	0.997
Insulin	$\log k = -55.93 + 8.05\pi^* + 42.90\alpha + 14.11\beta$	0.994
Calcitonin	$\log k = -16.40 + 20.41\pi^* + 32.36\alpha - 54.26\beta$	0.997

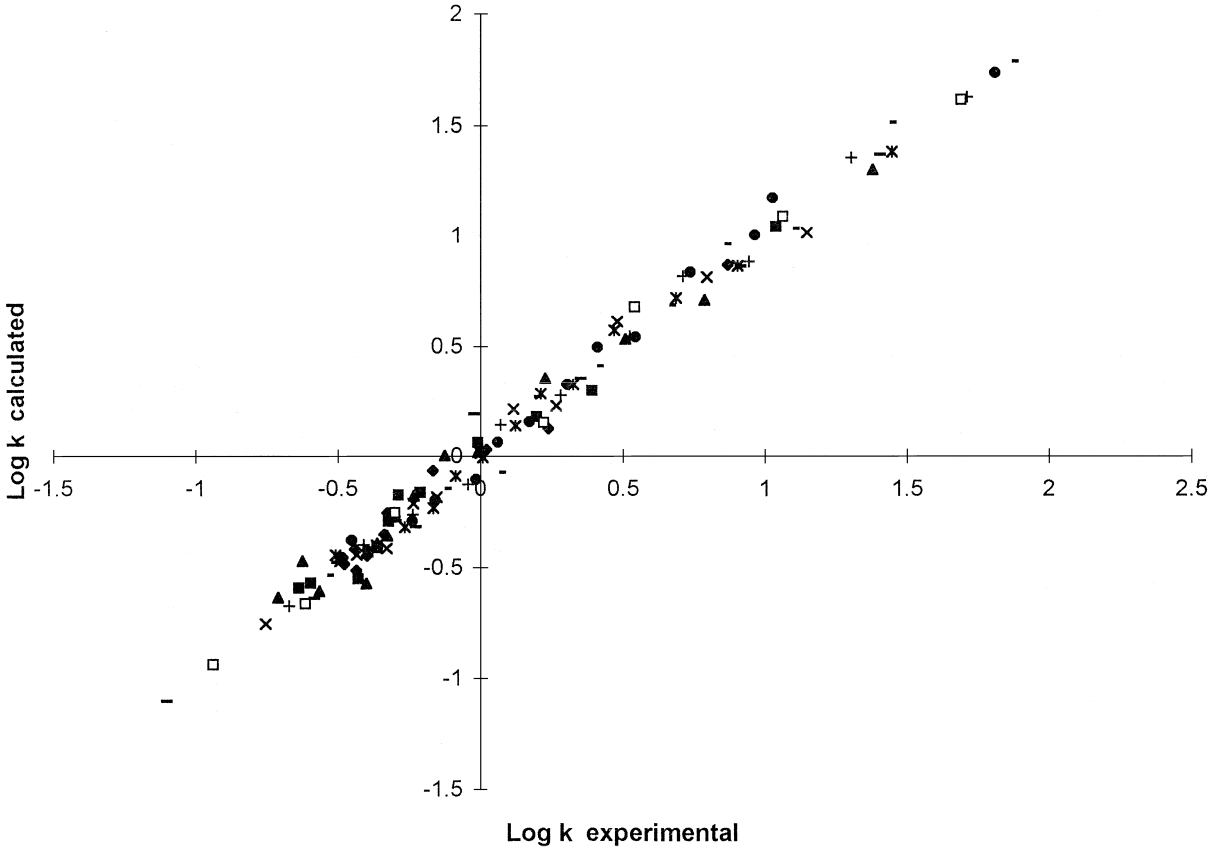

Fig. 1. Plot of log k values predicted with the LSER equations versus experimental log k values. VHLTPVEK (♦), lypressin (■), oxytocin (▲), bradykinin (×), Met-enkephalin (∗), Leu-enkephalin (●), triptorelin (+), bovine insulin (—), buserelin (-), and salmon calcitonin (□).

x_{ACN}, beyond which the acetonitrile molecules can no longer be accommodated within the cavities of the water structure, varies with the method applied, but is ≥ 0.10, approximately 25% (v/v) of acetonitrile. In the middle range of compositions, the acetonitrile–water mixtures show microheterogeneity; thus, there is a preference of a given water molecule for other water molecules rather than acetonitrile molecules. The same can be said for the preference of acetonitrile molecules in the vicinity of a given acetonitrile molecule. At $x_{ACN} \geq 0.75$ the number of water clusters is low and water–acetonitrile interactions, which could be discounted in the middle range, now become important.

In this study, two structural regions of acetonitrile–water mixtures has been used and, as a result, two straight lines with different slopes have been obtained, the change in the slope taking place in the region where the acetonitrile–water mixtures show microheterogeneity. This behaviour was also observed in previous studies [3,11,40] using different ranges of acetonitrile–water mixtures from 3 to 60% (v/v) of acetonitrile, and analysing different substances such as peptides, diuretics and quinolones, where plots of log k values versus the E_T^N parameters values showed two straight lines with different slopes, which intersect at acetonitrile percentages roughly from 15 to 30% (v/v). The study of higher percentages of acetonitrile in the case of these peptides is of no practical interest, since the resultant k values are subject to high errors due to the low retention, and there are difficulties in defining the column void volume.

The use of Eq. (3) involves an important reduction

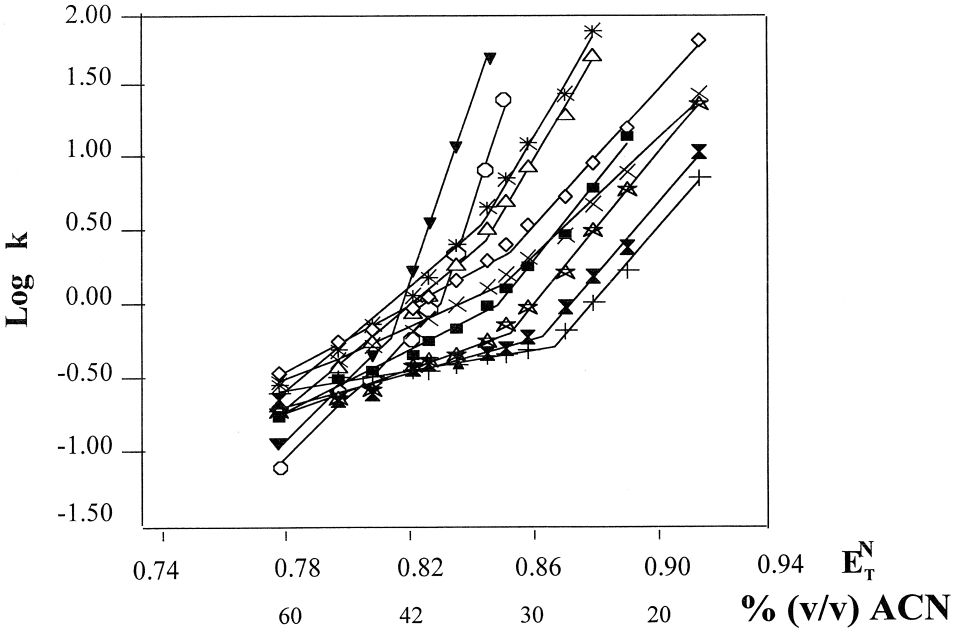

Fig. 2. Plot of the experimental log k values of the peptides studied versus the E_T^N parameter. VHLTPVEK (+), lypressin (⧗), oxytocin (☆), bradykinin (×), Met-enkephalin (■), Leu-enkephalin (◊), triptorelin (△), bovine insulin (○), buserelin (∗), and salmon calcitonin (▼).

in experimental work in the separation of complex peptide mixtures. The verified linearity of the log k values of peptides vs. E_T^N values, would be very useful for practical concerns in reference to the wide number of peptides that could exist in a given analysis. Thus, this behaviour could be extrapolated to other peptides and only two measurements of k values for each compound at two different percentages within one of the structural regions of the acetonitrile–water mixtures could be enough for predicting their retention behaviour and for optimising their chromatographic separation and resolution in a fixed stationary phase. Fig. 2 indicates that good chromatographic separation can be obtained for the peptides studied when the percentage of acetonitrile in the mobile phase is lower than approximately 35% (v/v).

The theoretical resolution between two adjacents peaks is given by:

$$R = \frac{1}{4}\sqrt{N} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k}{1+k}\right) \quad (14)$$

Efficiency Selectivity Retention

indicating that maximum resolution can be obtained by optimising the three terms, which are efficiency, selectivity and retention. Selection of the stationary phase as well as selection of the mobile phase components allows correct chromatographic behaviour, and good peak symmetry for all compounds to be obtained, providing optimum efficiency and retention.Therefore, selectivity defined as the relation between capacity factors for two consecutive peaks, $\alpha = k_2/k_1$, should be optimised to achieve an optimum resolution.

On the other hand, in order to examine the accuracy of retention predictions from Eq. (3), k values of peptides studied at different concentrations of acetonitrile ranging from 15% to 60% were calculated using Eq. (3), and selectivity for solute adjacent pairs was obtained. In Fig. 3 the lines indicate values obtained from Eq. (3) and the points show the selectivity values obtained from experimental data. As can be observed, experimental and estimated selectivity values agree in the whole range of acetonitrile contents studied.

Fig. 3 also indicates that good chromatographic separation in a reasonable retention time for the peptides studied can be achieved when the acetoni-

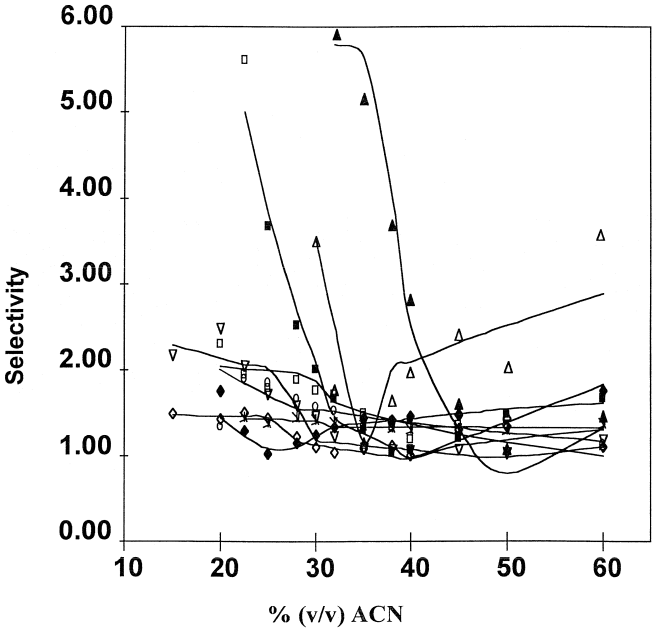

Fig. 3. Plot of the selectivity values of the peptides studied versus acetonitrile composition. Lypressin/VHLTPVEK (◊), oxytocin/lypressin (▽), bradykinin/oxytocin (□), Met-enkephalin/bradykinin (♦), Leu-enkephalin/Met-enkephalin (○), triptorelin/Leu-enkephalin (■), buserelin/triptorelin (∗), insulin/buserelin (▲), and calcitonin/insulin (△). Lines generated from Eq. (3) and points from experimental data.

trile content in the mobile phase is 32–35% (v/v). At lower proportions of organic modifier longer retention times are obtained; therefore a composition of 35% (v/v) was chosen for the separation of the peptide mixture studied.

On the other hand, in order to study the influence of the mobile phase pH on the chromatographic retention, *k* values for the peptides studied at different pH values of the mobile phase were determined from three different injections at every mobile phase pH considered, shown in Table 5. In the same table the standard deviation associated with k values have been included and a good repeatability can be observed. pH measurements in the hydroorganic mobile phase permit the interpretation of chromatographic results without extrapolation of pH values from aqueous solutions. pH and p*K* values show deviations from linear dependence on the composition of the mixtures because of preferential solvation [35,41]. Preferential solvation means that the solute interacts with one of the solvents more strongly than with the other [20].

From plots of *k* values for the peptides studied versus pH of the acetonitrile–aqueous phase eluent system [acetonitrile–water (35:65, v/v), 0.1% (v/v) TFA], shown in Fig. 4, the chromatographic behaviour of these peptide hormones can be predicted, depending on their acid–base properties. Peptides can be considered as typical zwitterion forming compounds [42]. However, the octadecylsilica (ODS) stationary phase can only be used in the pH range 2–7, so it was not 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. From a chromatographic point of view, with the widely used ODS stationary bonded phase, only the protolytic equilibria corresponding to p*K* values in the acid range are relevant. The octapeptide Val–His–Leu–Thr–Pro–Val–Glu–Lys presents two p*K* values in the acid range corresponding to dissociation of two carboxylic groups. In the case of Leu-enkephalin, Met-enkephalin and bradykinin only one p*K* value, corresponding to the dissociation of the terminal carboxylic group, has been considered. For triptorelin and buserelin the considered p*K* value corresponds to the dissociation of the protonated amino group from histidine. Lypressin and oxytocin

Table 5
Values of the capacity factors for the peptides studied at various pH values of the mobile phase in the acetonitrile–water (35:65, v/v) mixture, containing 0.1% (v/v) TFA used as eluent[a]

pH	*k*									
	VHLTPVEK	Lypressin	Oxytocin	Bradykinin	Met-Enkephalin	Leu-Enkephalin	Triptorelin	Buserelin	Insulin	Calcitonin
2.18	0.40 (0.01)	0.43 (0.01)	0.47 (0.01)	0.69 (0.01)	1.02 (0.01)	1.46 (0.01)	1.90 (0.01)	2.58 (0.01)	2.24 (0.01)	11.18 (0.04)
2.53	0.44 (0.01)	0.46 (0.01)	0.49 (0.01)	0.83 (0.01)	1.08 (0.01)	1.54 (0.01)	2.15 (0.01)	2.83 (0.01)	2.55 (0.01)	12.60 (0.01)
2.74	0.45 (0.01)	0.46 (0.01)	0.50 (0.01)	0.85 (0.01)	1.14 (0.01)	1.59 (0.01)	2.21 (0.01)	2.98 (0.01)	2.88 (0.01)	15.00 (0.01)
2.99	0.44 (0.01)	0.45 (0.01)	0.51 (0.01)	0.88 (0.01)	1.10 (0.01)	1.58 (0.01)	2.24 (0.01)	3.11 (0.01)	2.97 (0.01)	14.01 (0.01)
3.25	0.43 (0.01)	0.44 (0.01)	0.53 (0.01)	0.86 (0.01)	1.04 (0.01)	1.55 (0.01)	2.30 (0.01)	3.24 (0.01)	2.98 (0.01)	14.79 (0.01)
3.50	0.33 (0.01)	0.45 (0.01)	0.50 (0.01)	0.79 (0.01)	0.96 (0.01)	1.46 (0.01)	2.34 (0.01)	3.11 (0.01)	2.41 (0.41)	15.41 (0.01)
3.76	0.30 (0.01)	0.46 (0.01)	0.53 (0.01)	0.73 (0.01)	0.87 (0.01)	1.42 (0.01)	2.41 (0.01)	3.34 (0.01)	2.27 (0.01)	14.68 (0.01)
4.01	0.26 (0.01)	0.46 (0.01)	0.53 (0.01)	0.66 (0.01)	0.65 (0.01)	1.26 (0.01)	2.44 (0.01)	3.35 (0.01)	1.95 (0.01)	15.41 (0.01)
4.29	0.25 (0.01)	0.41 (0.01)	0.51 (0.01)	0.65 (0.01)	0.60 (0.01)	1.11 (0.01)	2.33 (0.01)	3.22 (0.01)	1.88 (0.01)	14.44 (0.01)
4.56	0.25 (0.01)	0.45 (0.01)	0.51 (0.01)	0.62 (0.01)	0.51 (0.01)	0.88 (0.01)	2.50 (0.01)	3.38 (0.01)	1.21 (0.01)	14.93 (0.01)
4.70	0.23 (0.01)	0.45 (0.01)	0.52 (0.01)	0.65 (0.01)	0.47 (0.01)	0.79 (0.01)	2.49 (0.01)	3.39 (0.01)	1.13 (0.01)	15.27 (0.01)
4.99	0.20 (0.01)	0.46 (0.01)	0.52 (0.01)	0.63 (0.01)	0.46 (0.01)	0.66 (0.01)	2.49 (0.01)	3.30 (0.01)	1.11 (0.01)	14.68 (0.01)
5.25	0.20 (0.01)	0.46 (0.01)	0.52 (0.01)	0.67 (0.01)	0.38 (0.01)	0.52 (0.01)	2.60 (0.01)	3.58 (0.01)	0.81 (0.01)	15.23 (0.01)
5.55	0.18 (0.01)	0.44 (0.01)	0.50 (0.01)	0.63 (0.01)	0.37 (0.01)	0.49 (0.01)	2.53 (0.01)	3.51 (0.01)	0.85 (0.01)	14.88 (0.01)
5.80	0.16 (0.01)	0.49 (0.01)	0.51 (0.01)	0.64 (0.01)	0.36 (0.01)	0.41 (0.01)	2.77 (0.01)	3.83 (0.01)	0.77 (0.01)	15.16 (0.01)
6.00	0.12 (0.01)	0.47 (0.01)	0.50 (0.01)	0.62 (0.01)	0.45 (0.01)	2.65 (0.01)	3.63 (0.01)	3.63 (0.01)	3.63 (0.01)	14.11 (0.01)
6.54	0.10 (0.01)	0.48 (0.01)	0.51 (0.01)	0.63 (0.01)	0.32 (0.01)	0.41 (0.01)	2.77 (0.01)	3.91 (0.01)	0.44 (0.01)	15.17 (0.01)
6.74	0.05 (0.01)	0.47 (0.01)	0.47 (0.01)	0.63 (0.01)	0.37 (0.01)	0.43 (0.01)	2.75 (0.01)	3.81 (0.01)	0.43 (0.01)	14.36 (0.01)

[a] Values in parentheses are standard deviation ($n=3$).

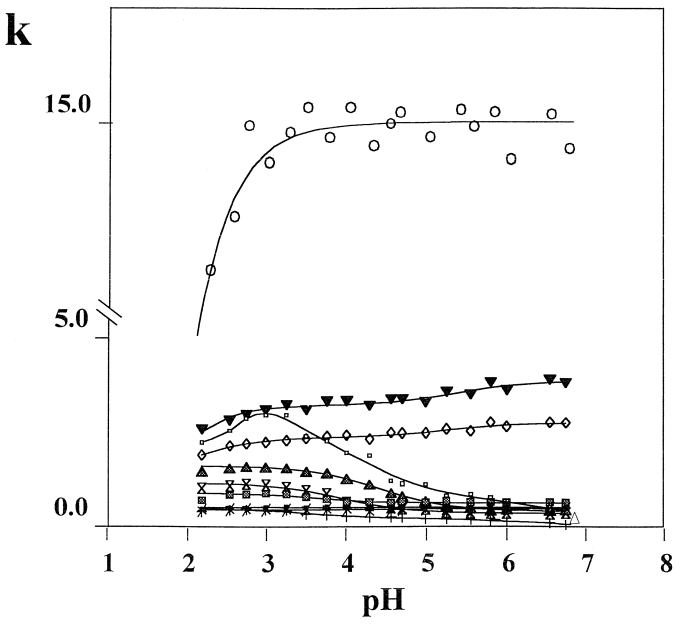

Fig. 4. Plot of the experimental *k* values of the peptides studied versus mobile phase pH in the acetonitrile–water (35:65) mixture with 0.1% (v/v) TFA used as eluent. VHLTPVEK (+), lypressin (∗), oxytocin (▪), bradykinin (■), Met-enkephalin (╳), Leu-enkephalin (▲), triptorelin (◊), bovine insulin (□), buserelin (▼), and salmon calcitonin (○). Lines are generated from Eqs. (6), (9) and (13) and points represent experimental data.

have protonated amino groups with p*K* values higher than 7 and no variation of chromatographic retention with pH was observed. In the case of bovine insulin and salmon calcitonin, the effect of solute ionization on retention has been studied, but the theoretical interpretation of this phenomenon is hampered by the lack of enough experimental data points for a rigorous treatment of excessive protolytic equilibria.

In this way, the retention of the peptides is high at low pH values, Fig. 4, where the peptide exists as a single (Met-enkephalin and Leu-enkephalin), double (triptorelin and buserelin), and triple (octapeptide and bradykinin) charged cation. When pH increases, the *k* value decreases and levels off at the isoelectric point pH and stays constant (Met-enkephalin, Leu-enkephalin and bradykinin). In the case of Val–His–Leu–Thr–Pro–Val–Glu–Lys the *k* value decreases again as the pH is increased where the two carboxylic acids are dissociated (Fig. 4). In the case of triptorelin and buserelin, the *k* value increases slightly due to the small dissociation of protonated amino groups when the pH increases.

The equations describing capacity factor as a function of mobile phase pH and the activity coefficient (Eqs. (6), (9) and (13)) [28] have twofold usefulness. Firstly, they can be effectively used to calculate the p*K* values of the substances and the capacity factors of the different species of the peptides, from the measured *k* values and the pH and activity coefficients variables. Secondly, these equations permit the prediction of the optimum eluting pH conditions from a minimum number of column *k*–pH–*y* measurements, which depend on the structure and the p*K* of the peptide. Each equation was experimentally verified and the p*K* values of the substances studied were determined from the experimental *k* values, the pH measurements and calculated activity coefficient values. The obtained p*K* values calculated by using a non-linear least-squares fit of the data to equations are listed in Table 6. The curves calculated using these equations are indicated as continuous lines and the plotted points are experimental data (Fig. 4).

On the other hand, from plots of *k* values for the peptides studied versus pH of the acetonitrile–aqueous phase eluent system [acetonitrile–water (35:65, v/v), 0.1% (v/v) TFA] shown in Fig. 4, it can be predicted that the optimal separation for the studied peptides could be achieved at pH of the mobile phase between 3 and 3.5.

Table 6
Chromatographic pK_a values of the peptides studied in the acetonitrile–water (35:65, v/v) mixture used as mobile phase[a]

Substance	pK_{a1}	pK_{a2}
Met-Enkephalin	3.96 (0.06)	
Leu-Enkephalin	4.38 (0.04)	
Bradykinin	3.21 (0.14)	
Triptorelin	4.71 (0.11)	
Buserelin	5.11 (0.11)	
VHLTPVEK	3.20 (0.25)	6.08 (0.14)

[a] Values in parentheses are standard deviations.

By injecting a mixture of the ten peptides with mobile phases pH adjusted to 3.21, 3.30, 3.42 and 3.59, the best separation was achieved at pH 3.30, with good resolution in all cases except between Val–His–Leu–Thr–Pro–Val–Glu–Lys and lypressin which were not resolved in these conditions. In Fig. 5 experimental resolution at every value of pH for the pairs of peptides which present problems in the separation is represented. A mobile phase of pH 3.3 was chosen, because of the maximum resolution

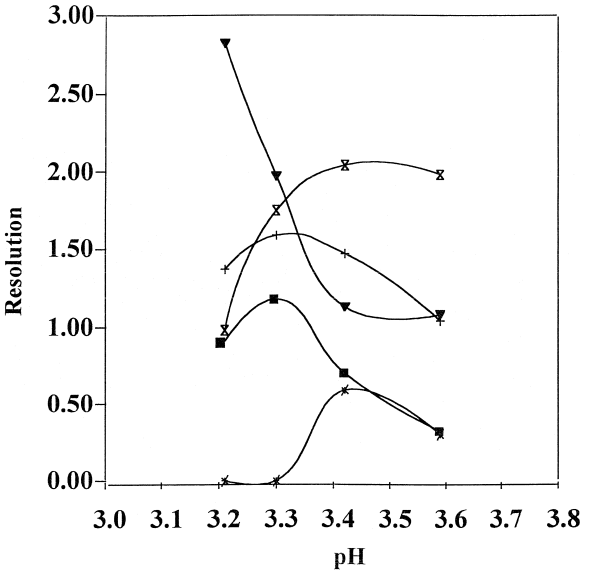

Fig. 5. Plot of experimental resolution versus the mobile phase pH. VHLTPVEK/lypressin (∗), lypressin/oxytocin (■), bradykinin/Met-enkephalin (+), triptorelin/insulin (▼) and insulin/buserelin (⧖).

between lypressin/oxytocin. The elution order was: Val–His–Leu–Thr–Pro–Val–Glu–Lys, lypressin, oxytocin, bradykinin, Met-enkephalin, Leu-Enkephalin, triptorelin, bovine insulin, buserelin, and salmon calcitonin. Fig. 6 shows the chromatogram of the separation of 10 peptides with an acetonitrile–water (35:65, v/v) plus 0.1% (v/v) trifluoroacetic acid as mobile phase at pH 3.30, the isocratic conditions which allow the separation of most of the peptides in the shortest elution time using reversed-phase liquid chromatography.

In conclusion, the verified linearity of the log k values of peptides vs. E_T^N values in the practical range of acetonitrile–water percentages permits, in many cases, the mobile phase optimisation, from only two log k values by using Eq. (3). On the other hand, if pK values are known, two measurements of k are enough for pH optimisation. This model can also be useful for the optimisation of gradient separation since it predicts the chromatographic behaviour of peptides at different percentages of acetonitrile.

Acknowledgements

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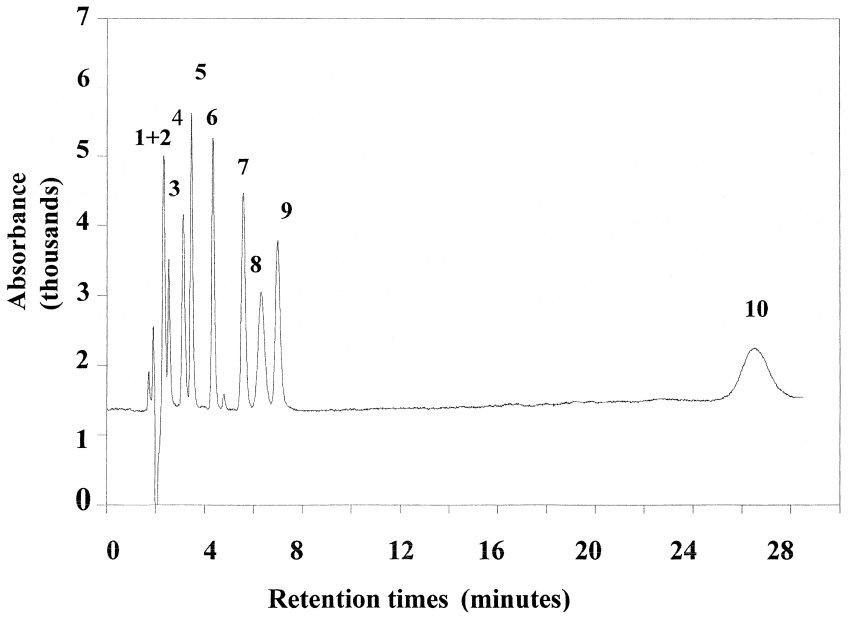

Fig. 6. Separation of VHLTPVEK+lypressin (1+2), oxytocin (3), bradykinin (4), Met-enkephalin (5), Leu-enkephalin (6), triptorelin (7), bovine insulin (8), buserelin (9), and salmon calcitonin (10), with a mobile phase consisting on acetonitrile–water (35:65, v/v), 0.1% (v/v) trifluoroacetic acid adjusted the mobile phase pH to 3.3 with sodium hydroxide.

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