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Analysis of a peptide hormone mixture of therapeutic interest by liquid chromatography coupled to high-flow pneumatically assisted electrospray mass spectrometry☆

I. Toro, V. Sanz-Nebot, J. Barbosa*

Departament de Química Analítica, Universitat de Barcelona, Diagonal 647, 08028 Barcelona, Spain

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Abstract

High-flow pneumatically assisted electrospray ionization mass spectrometry (ESI-MS) has been extensively used for the characterization and determination of peptides and peptide hormones available for biomedical research and therapeutic applications. The aim of this study was to optimize a method of characterization and determination of a mixture of peptide hormones with therapeutic interest by liquid chromatography (LC) coupled to ESI-MS. In this work the *linear solvation energy relationship* methodology was used in order to optimize the mobile phase to be used in the LC separation of the peptide hormone series and the operational parameters of the source and analyzer of ESI were also optimized to obtain the best signal stability and the highest sensitivity. To validate the proposed method for peptide hormone analysis, quality parameters were determined and satisfactory results were obtained. Likewise, the method detection limit was picomole level for most of the peptides employing selected-ion monitoring of the $[M+nH]^{n+}$ ions.

Keywords: Linear solvation energy relationships; Peptides; Hormones

1. Introduction

Over the last few years, high-flow assisted electrospray ionization mass spectrometry (ESI-MS) has been extensively used for the characterization and determination of peptides and peptide hormones available for biomedical research and therapeutic applications [1–5]. The combination of liquid chromatography (LC) with mass spectrometry allows efficient separation and identification of components in mixtures with greater specificity than can be obtained using liquid chromatography and ultraviolet detection.

The main goal of this work is the establishment of a methodology of analysis of commercially available peptide hormones by liquid chromatography coupled to electrospray mas spectrometry in order to apply this method to the analysis of the synthesis crudes of peptides and other biological peptide samples. In this way, in our work, we propose a quantitative method easy to use for an increasing number of new and challenging biomedical problems call.

Liquid chromatography (LC) coupled to ESI-MS, not only allows molecular masses to be measured

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*Corresponding author. Tel.: +34-93-4021-797; fax: +34-93-402-1233.

E-mail address: isator@zeus.qui.ub.es (J. Barbosa).

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with good accuracy, but also allows specific fragments be generated from individual compounds in a mixture that can help to verify or deduce chemical structures. On the other hand, the ability of ESI to generate multiply charged species is, of course, one of its attractive features because it greatly extends the effective mass range of the ESI-MS system. Thus, LC–ESI-MS has become the coupling method of choice for the analysis of peptides and peptide hormones [6].

In order to avoid long empirical optimization procedures, a system for predicting peptide retention times is of great advantage. *Linear solvation energy relationship* (LSER) formalism has been used successfully to study retention in LC [7–10] and seeks to relate chromatographic retention to variations in characteristic properties of mobile phases such as the solvatochromic parameter π^*, that evaluates solvent dipolarity/polarizability [11] and parameters α, β, that evaluate solvent hydrogen-bond acidity [12] and solvent–hydrogen bond basicity [13] respectively. The general equation is:

$$\begin{aligned}\text{Log } k = (\log k)_0 &+ M(\delta_s^2 - \delta_m^2)\bar{V}_2/100 \\ &+ S(\pi_s^* - \pi_m^*)\pi_2^* + A(\alpha_s - \alpha_m)\beta_2 \\ &+ B(\beta_s - \beta_m)\alpha_2 \qquad (1)\end{aligned}$$

Here, k is the chromatographic capacity factor; $(\log k)_0$ is the intercept of the regression equation, $\bar{V}_2$ is the molar volume of the solute, δ^2 is the square of the Hildebrand solubility parameter (a measure of the solvent cohesive energy). Subscripts s and m denote the stationary and mobile phases respectively and subscript 2 refers to the solute properties. The values of M, S, A and B are the correlation coefficients for this equation and do not depend on the solute [14]. 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 [14] and the relationship between δ^2 and the solvatochromic parameters of the mobile phase [14,15], Eq. (1) can be simplified to:

$$\text{Log } k = (\log k)_0 + s\pi_m^* + a\alpha_m + b\beta_m \qquad (2)$$

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 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 [1,10,16,17]. Taking into account that β values of acetonitrile are nearly constant over most of the composition range [18,19], and the observed correlation between the normalized Dimroth and Reichardt polarity parameter, E_T^N [20], and π^* and α solvatochromic parameters, $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [19], Eq. (2) can be reduced to a single solvent parameter-dependent expression:

$$\text{Log } k = C + eE_T^N \qquad (3)$$

Eq. (3) has been widely used to correlate chromatographic retention of different solutes with the polarity of the mobile phases [1,10,16,17,21,22], 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.

In this work the optimization of the hydroorganic mobile phase, used for the chromatographic separation was performed using LSER methodology. The verified linearity of $\log k$ versus E_T^N parameter previously demonstrated [1,10,16,17,22], allows the elution behaviour of the peptide hormones to be predicted from only three experimental measurements of k values. We have used LC coupled to high-flow pneumatically assisted electrospray for the separation, characterization and determination of peptide hormones with molecular masses ranging from 500 to 1600. The optimization of the different operating parameters such as sample cone voltage, counter-electrode (high-voltage lens) voltage, ion energy and source temperature was performed [2,23] in order to obtain the best signal and the highest sensitivity for the peptide hormones studied. The LC–ESI-MS method proposed for peptide hormone analysis established the quality parameters: repeatability, reproducibility, linearity range, limit of detection (LOD) and limit of quantitation (LOQ).

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm

and acetonitrile (Merck, Darmstadt, Germany) were of LC grade. Trifluoroacetic acid (TFA), potassium bromide and potassium hydrogenphthalate were all analytical grade obtained from Merck. The peptides used in this study were purchased: Met-enkephalin, Leu-enkephalin from Sigma Química (Alcobendas, Madrid, Spain), buserelin from Hoechst Ibérica (Barcelona, Spain), triptorelin from Lasa (Barcelona, Spain) and desmopressin, carbetocin, eledoisin and somatostatin from Lipotec (Barcelona, Spain). A summary of the hormone peptides studied here, listing their amino acid sequence and molecular weight is 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 −4°C when not in use. Stock solutions of the peptides were prepared by dissolving approximately 5 mg of each peptide and diluting to 5 ml; working solutions were prepared by ten-fold dilution of each stock solution respectively. The mixtures of the nine peptides studied were prepared by 10–100-fold dilution of the stock solutions and by 100-fold dilution of the working solutions.

2.2. Apparatus

LC–ESI-MS measurements were performed using two Phoenix 20 syringe pumps (Carlo Erba, Milan, Italy) with a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 10-μl sample loop, coupled to a VG Platform II single quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a nebulizer-assisted electrospray source.

The separation was performed on a Spherisorb ODS2 narrow-bore (5 μm) column 150×2.1 mm I.D. (Tecknokroma, Barcelona, Spain) at ambient temperature and with a flow-rate of 100 μl/min. Instrument control and data analysis were performed using MASSLYNX application software from Micromass. Generally the mass spectral data are the average of three separate measurements. 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, Boston, MA, USA).

2.3. Procedures

2.3.1. Optimization of the operational parameters of the mass spectrometer

For the optimization of the source and analyzer parameters of ESI-MS, three peptide hormones were chosen: Met-enkephalin, carbetocin and buserelin to cover the molecular mass range from 500 to 1600. The optimization of the operational parameters was performed using 50 μg/ml solutions of each selected peptide, that corresponds to molar concentrations ranging from 30 to 80 pmol/μl approximately, and acetonitrile–water (40:60, v/v) with 0.05% TFA as eluent. Each peptide hormone selected was introduced directly into the ESI source, at a flow-rate of 100 μl/min and working in full scan mode of data acquisition.

2.3.2. Optimization of the mobile phase

For the optimization of the mobile phase composition, eluents were acetonitrile–water mixtures containing 0.05% (v/v) TFA [24,25], pH approxi-

Table 1
International name, amino acid sequence and molecular mass (*M*) of the peptide hormones studied

International name	Amino acid sequence	*M*
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu	555.2
Met-enkephalin	Tyr-Gly-Gly-Phe-Met	573.7
Carbetocin	$CO\text{-}CH_2\text{-}CH_2\text{-}CH_2$-------S Tyr(OMe)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 cyclic (1–6) disulfide	988.2
Bradykinin	Arg-Pro-Pro-Gly-Ser-Pro-Phe-Arg	1060.2
Desmopressin	$CO\text{-}CH_2\text{-}CH_2S$-----S Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-Gly-NH_2 cyclic (1–6) disulfide	1069.2
Eledoisin	<Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2	1188.4
Buserelin	<Glu-His-Trp-Ser-Tyr-D-Ser(*tert*.-Bu)-Leu-Arg-Pro-NHC_2H_5	1239.7
Triptorelin	<Glu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2	1311.5
Somatostatin	Ala-Gly-Cys-Lys-Asn-Phe-Trp-Arg-Pro-Thr-Ser-Cys cyclic (3–14) disulfide	1637.9

mately 2.2, at several proportions of organic solvent from 32 to 38% (v/v). The aqueous component of the eluent was filtered through a 0.45-μm filter (MSI, Scharlau, Barcelona, Spain) before use. Mobile phases were flushed under a stream of helium and the flow-rate was maintained at 100 μl/min.

The hold-up time, t_0, was established for every mobile phase tested by injection of a 0.01% (w/v) potassium bromide solution in water [26] and monitoring the eluate at m/z 39 in positive ionization (PI) mode. The retention times and the retention factors for all the peptide hormones at each mobile phase composition were determined from three different injections of the compound solutions, which were filtered through a 0.22-μm filter (MSI). TIC (*total ion current)* chromatograms and ESI–MS spectra were obtained at optimum conditions of the mass spectrometer and working in full scan mode (m/z 250 to 1750) of data acquisition and positive mode of ionization.

The pH of the mobile phase was measured by using a 0.05 mol/kg potassium hydrogenphthalate solution as primary standard buffer reference dissolved in the appropriate acetonitrile–water medium [27,28] and a commercial combination pH electrode as described in previous works [10,16,29].

2.3.3. ESI-MS spectra of peptide hormones

In order to characterize the different peptide hormones, a solution of each compound was injected into the LC–ESI-MS system. The narrow-bore C_{18} column was equilibrated for 30 min with the optimized mobile phase acetonitrile–water containing 0.05% (v/v) TFA. ESI-MS spectra of each peptide hormone were obtained at optimum conditions of the mass spectrometer and working in full scan mode of data acquisition and positive mode of ionization.

2.3.4. Quality parameters

The repeatability ($n=5$) and the long-term reproducibility ($n=15$) were determined by injecting the peptide hormone mixture at individual concentrations ranging from 10 to 150 pmol/μl in the above optimized experimental conditions, five times in one day and for three different days respectively. The repeatability and long-term reproducibility have been calculated as percent of relative standard deviation (RSD, %) of retention times and areas obtained. The data acquisition was in both cases full scan mode.

The range of linearity has been established by injecting a solution of the peptide hormone mixture with individual concentrations ranging from 0.4 to 200 pmol/μl depending on the peptide. The LODs were calculated by using a signal-to-noise ratio of 3 (the ratio between the peak intensity using selected ion monitoring (SIM) conditions and the intensity of the noise was used). The LOQs were calculated by using a signal-to-noise ratio of 10 (the ratio between the peak intensity in SIM conditions and the intensity of the noise was used). The data acquisition for the determination of linearity range, LODs and LOQs was in SIM mode of $[M+nH]^{n+}$ ions.

3. Results and discussion

Source and analyzer parameters of ESI-MS system were optimized in order to obtain the best signal and the highest sensitivity of the peptide hormones. Likewise, the percentage of relative response for the three representative peptides selected (Met-enkephalin, carbetocin and buserelin), varying the cone voltage is shown in Table 2. The buserelin appears to have, at the same molar concentration, better response than carbetocin and Leu-enkephalin; more-

Table 2
Values of the percentage of relative response normalized by an equimolar concentration for the three selected peptide hormones, Leu-enkephalin, carbetocin and buserelin at sample cone voltages of 30, 50, 70, 90 and 110 V[a]

Cone voltage (V)	Relative response (%)		
	Leu-enkephalin	Carbetocin	Buserelin
30	9.6	3.7	11.5
50	17.3	12.2	43.0
70	15.1	22.6	68.9
90	15.7	22.5	89.7
110	16.8	19.0	100.0

[a] Eluent: MeCN–water (40:60), 0.05% (v/v) TFA.

Table 3
Values of the logarithm of the retention factors of the peptides studied and the E_T^N values at various percentages of acetonitrile in the mobile phase

MeCN (%)	E_T^N	Log k								
		Desmopressin	Carbetocin	Eledoisin	Met-enkephalin	Leu-enkephalin	Bradykinin	Triptorelin	Buserelin	Somatostatin
32	0.8	−0.384	−0.019	0.168	−0.014	0.193	0.227	0.680	0.831	0.950
37	0.8	−1.000	−0.753	−0.274	−0.257	−0.023	0.183	0.328	0.498	0.637
38	0.8	−1.160	−0.891	−0.366	−0.305	−0.097	0.176	0.273	0.460	0.599

over, the response increased if the cone voltage is increased, especially for the buserelin; in the case of Leu-enkephalin, the response appears to be constant in the range of cone voltage studied. The cone voltage of 90 V was selected since the carbetocin response decreased at a higher cone voltage. Another point to consider from this result is that, at this selected cone voltage, more structural information is obtained as compared to a lower cone voltage, and consequently, this higher voltage will be recommended when characterization is required. The other parameters were optimized in the same way, maintaining the previously optimized parameters constant. Then, optimum values of the different operating parameters were: ES voltage 3.5 kV, counter-electrode voltage 0.5 kV, sample cone voltage 90 V, ion energy 3.0 V and source temperature 80°C.

For the optimization of the mobile phase composition, the logarithm of the retention factor values (log k) were obtained using acetonitrile–water mixtures at three percentages of acetonitrile, 32, 37 and 38% by volume, for the peptide hormone series. The Reichardt's E_T^N polarity parameter values (known for the whole range of compositions of acetonitrile–water) [30] (Table 3), were related with the retention factors of the peptide hormones studied, using the LSER methodology. All the peptide hormones showed a similar elution profile. Log k and E_T^N correlate linearly (correlation coefficients greater than 0.999) over the whole experimental range of acetonitrile contents studied. Fig. 2 shows plots of log k of the peptide hormones versus E_T^N values. This behaviour can be explained taking into account the structural features of the acetonitrile–water mixtures

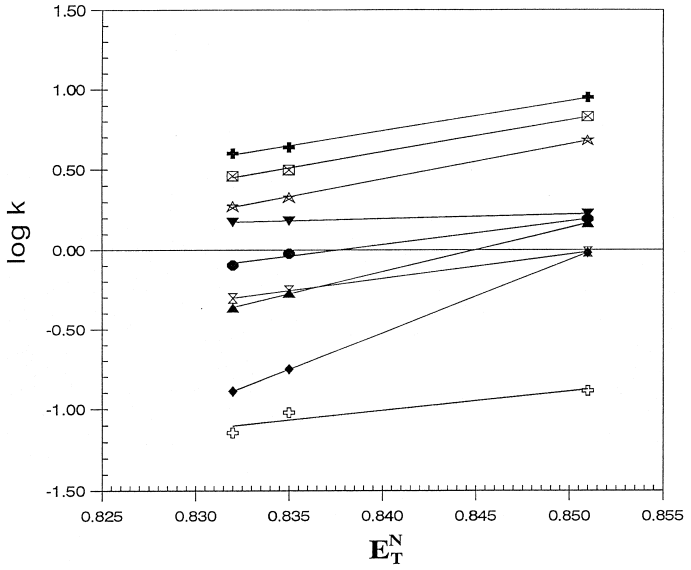

Fig. 1. Plots of log k of the peptide hormone mixture studied versus E_T^N parameters of the mobile phase. Symbols: (✚) Somatostatin, (⊠) buserelin, (☆) triptorelin, (▼) bradykinin, (●) Leu-enkephalin, (⊠) Met-enkephalin, (▲) eledoisin, (♦) carbetocin and (✣) desmopressin.

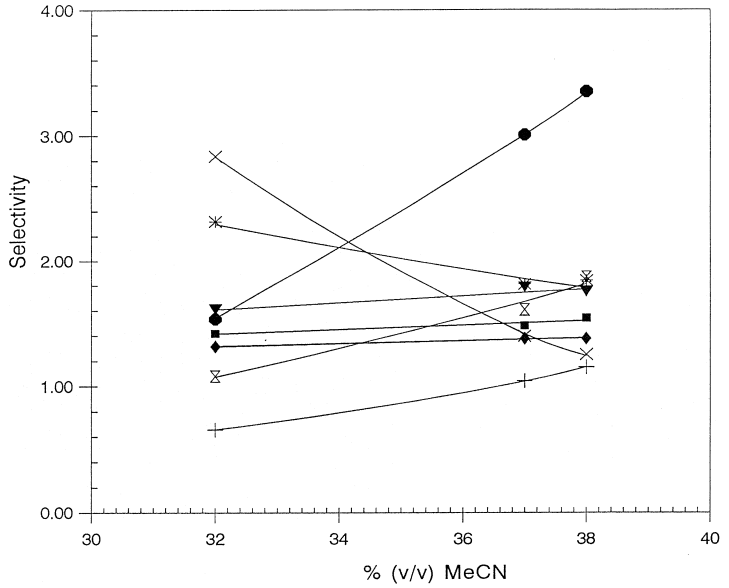

Fig. 2. Variation in selectivity values for compound pairs with acetonitrile percentage. Solid lines indicate predicted values of selectivity from Eq. (3) and points represent experimental values of selectivity: (✳) carbetocin/desmopressin, (●) eledoisin/carbetocin, (+) Met-enkephalin/eledoisin, (▼) Leu-enkephalin/Met-enkephalin, (⧖) bradykinin/Leu-enkephalin, (×) triptorelin/bradykinin, (■) buserelin/triptorelin, (♦) somatostatin/buserelin.

[19] considered in previous papers [1,10,16]. In this study, we have worked in one structural region of acetonitrile–water mixtures, and as was expected, we have obtained one straight line for every peptide hormone.

Therefore, suitable prediction of the retention for a specific solute can be achieved considering E_T^N values of the mobile phase and a few k experimental data. In our case, only three experimental data for each substance studied were used to correlate $\log k$ versus E_T^N values of MeCN–water media considered as it is shown in Fig. 1.

The use of the linear relationships of $\log k$ versus E_T^N values, Eq. (3), involves an important reduction in experimental work in the separation of complex peptide mixtures. The verified linearity between the two parameters, would be very useful for practical concerns in reference to the wide number of peptides that could exist in a given mixture.

In order to examine the accuracy of retention prediction using Eq. (3), the selectivity was obtained for adjacent solute pairs with separation problems, in the usual way, $\alpha = k_i/k_j$. Fig. 2 shows variation of

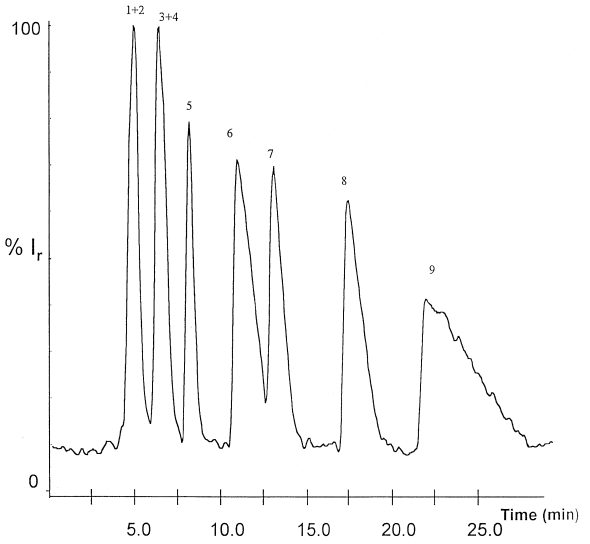

Fig. 3. Total ion current (TIC) chromatogram of a solution containing the mixture of peptide studied, with a mobile phase of acetonitrile–water (38:62, v/v), 0.05% TFA. Mode of data acquisition in full scan. (1) Desmopressin, (2) carbetocin, (3) eledoisin, (4) Met-enkephalin, (5) Leu-enkephalin, (6) bradykinin, (7) triptorelin, (8) buserelin and (9) somatostatin.

selectivity for the solute pairs versus percentage of acetonitrile in the mobile phase. Solid lines indicate theoretical selectivity values obtained using Eq. (3), and points represent experimental selectivity values. Fig. 2 shows that values of α are concordant from the two methods. Thus it appears clear that three measurements per compound of k are enough to predict the optimum mobile phase composition, in accordance with the results obtained in previous works [1,11,17].

From Figs. 1 and 2 we can concluded that the optimum chromatographic separation between the peptide hormone present in the mixture can be achieved at percentages of acetonitrile in the mobile phase from 37 to 38% (v/v). The mobile phase composition of 38% was chosen because of the better selectivity values for the most of the peptides and shorter analysis times.

A TIC chromatogram of the peptide hormone series at optimal conditions of mobile phase composition and of different operating parameters of the ESI-MS system, is given in Fig. 3. Good separation is achieved in all cases except between the pairs desmopressin/carbetocin and eledoisin/Met-enkephalin, which are not resolved under these conditions.The order of elution is: desmopressin, carbetocin, eledoisin, Met-enkephalin, Leu-enkephalin, bradykinin, triptorelin, buserelin and somatostatin.

The electrospray mass spectra has been obtained by injecting individually in the optimal conditions each peptide hormone. The spectra obtained for the triptorelin and buserelin have been illustrated as an example in Fig. 4, as well as the structural assignments for the observed fragments of these peptide hormones according to the nomenclature of Roepstorff et al. [31].

Table 4 summarizes the most relevant m/z values observed with the relative intensities of the peaks between parentheses, the structural assignments of respective charged forms and the number of basic

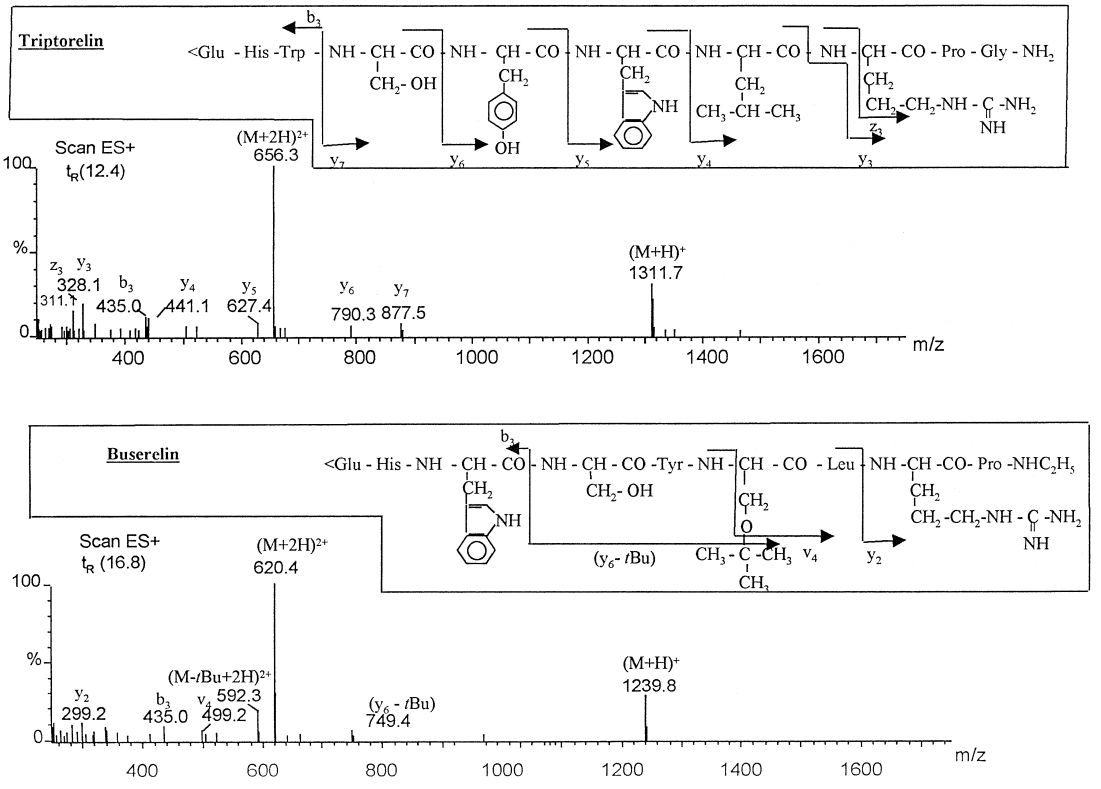

Fig. 4. Electrospray mass spectra of triptorelin and buserelin associated with the TIC peaks at the retention time indicated: (a) 12.4 mm and (b) 16.8 mm. Experimental conditions indicated in Fig. 3.

Table 4
Results obtained by LC–ESI-MS: mass-to-charge ratios (m/z) with relative intensities in parentheses, structural assignments, basic residues and observed fragments according to the nomenclature of Roepstorff et al. [31]

Substance	m/z of major ions (abundance)	Interpretation	Basic residues	Fragments
Desmopressin	1069.5 (100) 535.3 (90)	$(M+H)^+$ $(M+2H)^{2+}$	1 Arg	y_3, y_3-NH_3
Carbetocin	285.2 (100) 988.6 (84) 704.3 (80)	Fragment y_3 $(M+H)^+$ Fragment b_6	–	y_3, z_4, c_5, a_6, b_6, b_9, a_6-NH_3
Eledoisin	1188.9 (100) 594.8 (80)	$(M+H)^+$ $(M+2H)^{2+}$	1 Lys 1 Met	c_3, b_5, b_6, y6, b_{10}, b_5-H_2O
Met-enkephalin	574.1 (100)	$(M+H)^+$	1 amino N-terminus	y_2, b_3, a_4, b_4
Leu-enkephalin	556.2 (100)	$(M+H)^+$	1 amino N-terminus	z_2, b_3, z_3, a_4, b_4
Bradykinin	530.8 (100) 1060.7 (20)	$(M+2H)^{2+}$ $(M+H)^+$	2 Arg 1 amino N-terminus	y_3, y_2
Triptorelin	656.3 (100) 1311.7 (30)	$(M+2H)^{2+}$ $(M+H)^+$	1 Arg I His	b_3, y_3, z_3, y_4, y_5, y_6, y_7
Buserelin	620.4 (100) 1239.8 (45)	$(M+2H)^{2+}$ $(M+H)^+$	1 Arg I His	y_2, b_3, v_4, y_6-tBu
Somatostatin	819.6 (100) 546.7 (15) 1638.0 (12)	$(M+2H)^{2+}$ $(M+3H)^{3+}$ $(M+H)^+$	2 Lys 1 amino N-terminus	$[Trp\text{-}Lys\text{-}NH_3]^+$

residues present in the primary structure of each peptide hormone responsible of most of the charges of the peptide hormone. Likewise, the m/z values observed for the fragments of each peptide hormone in the electrospray mass spectra were indicated with the corresponding name according the nomenclature of Roepstorff et al. [31]. The electrospray mass spectra associated with the other peptide hormone were shown in Fig. 5.

The electrospray mass spectra associated with triptorelin and buserelin, Fig. 4, $t_R = 12.4$ min and $t_R = 16.8$ min respectively, show the ion corresponding to the $[M+H]^+$ (triptorelin: m/z 1311.7 and buserelin: m/z 1239.8) and the ion corresponding to the $[M+2H]^{2+}$ (triptorelin: m/z 656.3 and buserelin: m/z 620.4) and a series of minor signals corresponding to fragments of the each peptide hormone.

The ions selected to work in mode of data acquisition SIM were corresponding to the molecular species of each peptide with different charges, $[M+nH]^{n+}$ ions, ranging n from 1 to 3, depending on the peptide. The molecular mass obtained for the peptide hormone studied from the corresponding spectrum, agree well with theoretically value (RSD$<$0.09%). The experimental molecular mass values, M, were calculated using an averaging algorithm of MASSLYNX software, from the set of m/z peaks of spectrum corresponding to the series of charged states of each molecular species.

In order to calculate repeatability, five replicate analyses of standard solutions of a peptide hormone mixture with individual concentrations ranging from 10 to 150 pmol/μl of each substance were carried out under the optimum conditions. Relative standard deviations (%RSD) based on peak area and retention time in the range from 0.2 to 9% were obtained.

In order to test the day-to-day reproducibility, five replicate analyses of a peptide hormone mixture with individual concentrations ranging from 10 to 150

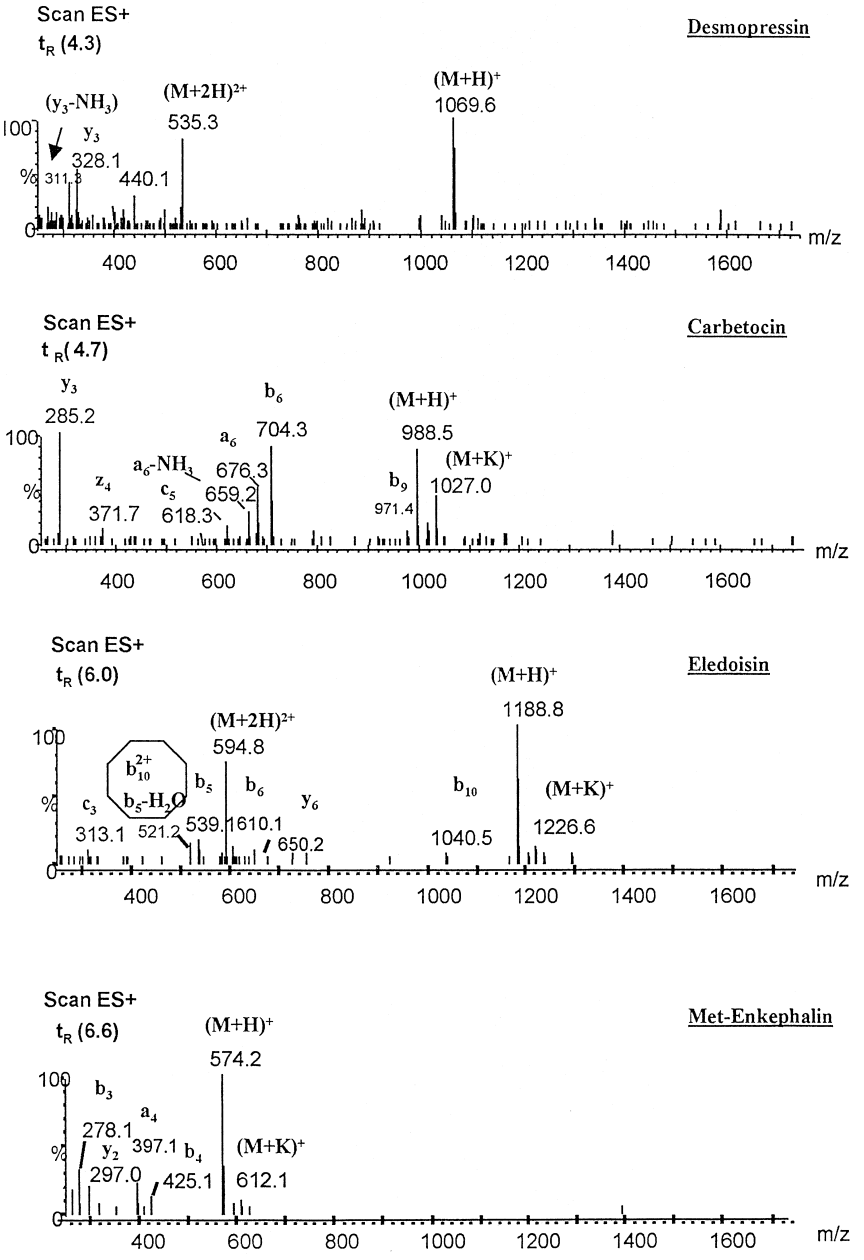

Fig. 5. Electrospray mass spectra of the other studied peptide hormone associated with the TIC peaks at the retention time indicated. Experimental conditions as indicated in Fig. 3.

pmol/μl of each substance were carried out under the optimum conditions on three different days. RSDs based on peak area and retention time in the range from 1.5 to 20% were obtained.

Repeatability and reproducibility were obtained working in data acquisition mode of full scan. It has been reported by others authors that the reproducibility often exceeds 20%. The range of the values obtained by us is slightly better for most of the peptide hormones [7,32], Table 5. The quantitation

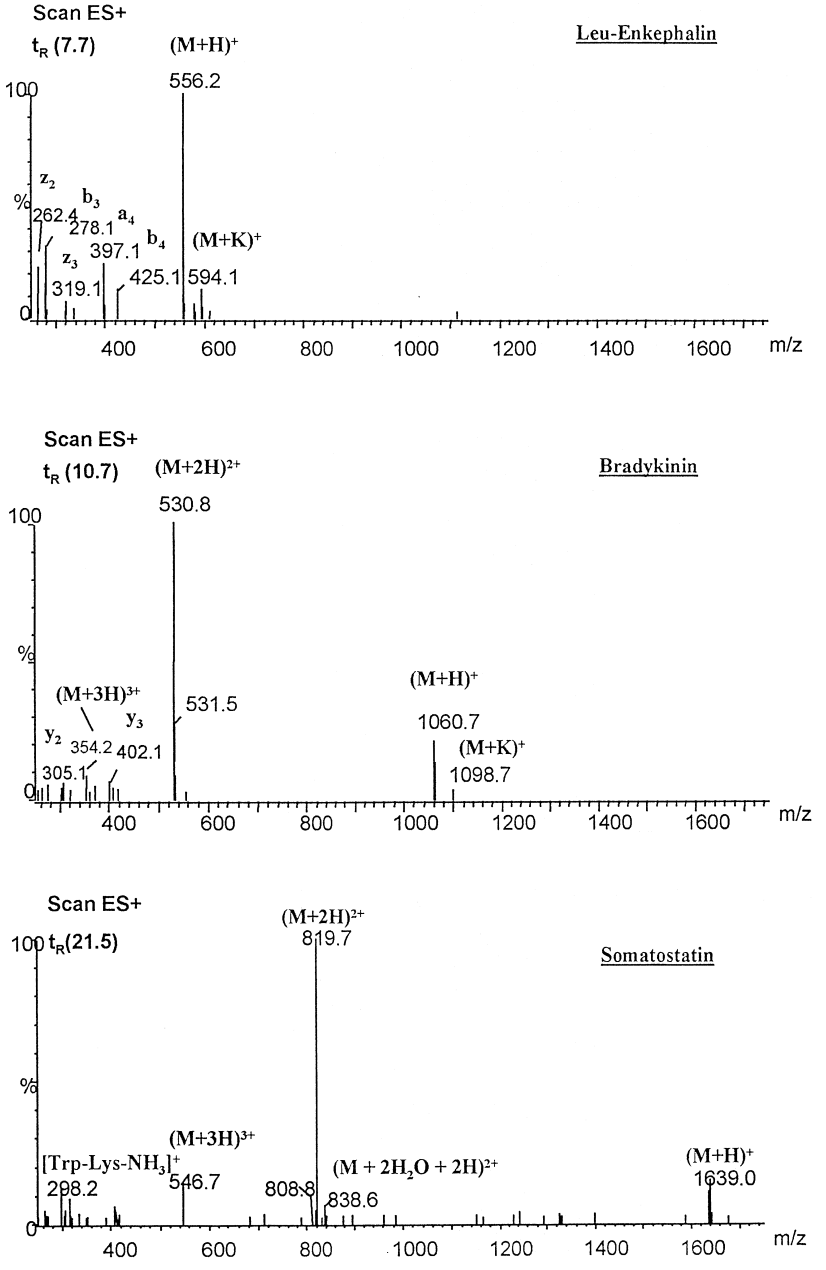

Fig. 5. (*continued*).

was performed by external calibration, plotting peak area versus concentration injected into the system using positive ion mode and SIM of the $[M+nH]^{n+}$ ions. The calibration equations are shown in Table 6 (coefficient correlations of 0.999). The system was linear in all the cases using seven points in the range from 0.4 to 200 pmol/μl approximately depending on the peptide hormone.

The LOD and LOQ were calculated by using a signal-to-noise ratios of 3 and 10 respectively,

Table 5
Limit of detection (LOD) and limit of quantification (LOQ) for the peptide hormone mixture studied using SIM of the $[M+nH]^{n+}$ ions[a]

Substance	LOD (pmol/μl)	LOQ (pmol/μl)	RSD (%) (1) $n=5$	RSD (%) (1) $n=15$	RSD (%) (2) $n=5$	RSD (%) (2) $n=15$
Desmopressin	0.46	1.53	1.5	1.6	4.3	13.6
Carbetocin	0.42	1.40	0.3	2.9	9.0	10.7
Eledoisin	0.57	1.90	0.3	3.6	0.6	10.3
Met-enkephalin	0.57	1.90	1.6	1.9	4.9	10.2
Leu-enkephalin	0.77	2.56	3.4	2.4	4.7	10.1
Bradykinin	1.13	3.77	3.0	3.4	7.6	8.6
Triptorelin	0.50	1.67	3.3	3.1	4.5	20.2
Buserelin	0.51	1.70	3.5	5.1	4.4	19.7
Somatostatin	0.87	2.90	3.9	5.8	2.1	16.9

[a] Values of repeatability (RSD, $n=5$) and reproducibility (RSD, $n=15$) of retention times (1) and areas (2) respectively.

Table 6
Calibration data and correlation coefficients (r) for the peptide hormone mixture studied using SIM of the $[M+nH]^{n+}$ ions

Substance	Linear equation (pmol/μl)	r
Desmopressin	$A=746.5+1303.8C$	0.999
Carbetocin	$A=1153.3+1051.0C$	0.999
Eledoisin	$A=671.7+1706.3C$	0.999
Met-enkephalin	$A=1404.4+903.4C$	0.997
Leu-enkephalin	$A=1140.9+1317.5C$	0.999
Bradykinin	$A=7685.5+2074.7C$	0.999
Triptorelin	$A=1091.0+831.0C$	0.999
Buserelin	$A=3787.7+3058.4C$	0.999
Somatostatin	$A=2194.9+4859.3C$	0.999

employing SIM of the $[M+nH]^{n+}$ ions (Table 5) and the method detection limit was of the picomole level for most of the peptide hormones. A chromatogram of the peptide hormone mixture at the optimal conditions working in SIM at a concentration level close to the detection limits is shown in Fig. 6.

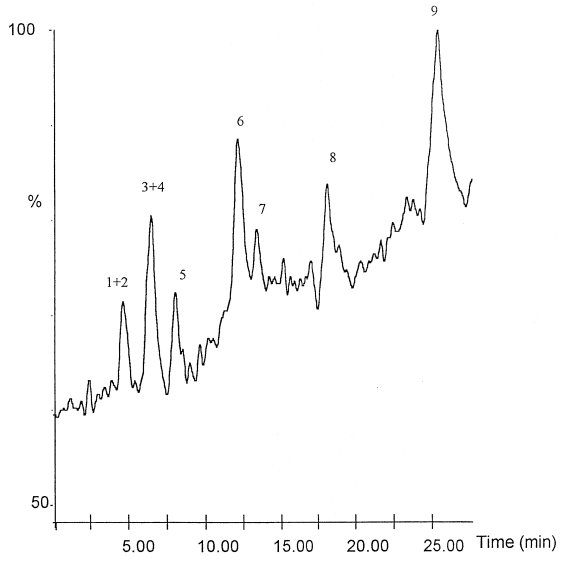

Fig. 6. LC–ESI-MS–SIM chromatogram of a solution containing the mixture of the studied peptides at a concentration level close to each peptide hormone detection limit. Mobile phase and numbers indicated in Fig. 3.

4. Conclusions

We can conclude that the verified linearity of the $\log k$ values of the peptides versus E_T^N values in the practical range of acetonitrile–water ratios allows the mobile phase to be optimized from only three $\log k$ values, using Eq. (3), and this involves an important reduction in experimental work in the separation of complex peptide hormone mixtures. Furthermore, the combination of liquid chromatography and electrospray ionization mass spectrometry provides an efficient tool for characterization and determination of the peptide hormone series, which is representative of the complex media commonly encountered in the usual peptide analysis.

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References

[1] V. Sanz-Nebot, I. Toro, J. Barbosa, J. Chromatogr. A 846 (1999) 25.
[2] V. Sanz-Nebot, A. Garcés, J. Barbosa, J. Chromatogr. A 833 (1999) 267.
[3] L. Rovatti, O. Curcuruto, M. Hamdan, E. Cassano, C. Galoppini, P. Rovero, Rapid Commun. Mass Spectrom. 10 (1996) 1504.
[4] R.D. Smith, J.A. Loo, C.G. Edmonds, C.J. Barinaga, H.R. Udseth, Anal. Chem. 62 (1990) 882.
[5] A. Casazza, O. Curcuruto, M. Hamdan, A. Bisello, E. Peggion, J. Chromatogr. A 715 (1995) 227.
[6] S.J. Gaskell, J. Mass Spectrom. 32 (1997) 677.
[7] P.C. Sadek, P.W. Carr, R.M. Doherty, M.J. Kamlet, R.W. Taft, M.H. Abraham, Anal. Chem. 57 (1985) 2971.
[8] P.W. Carr, R.M. Doherty, M.J. Kamlet, R.W. Taft, W. Melander, Cs. Horváth, Anal. Chem. 58 (1986) 2674.
[9] J.H. Park, P.W. Carr, M.H. Abraham, R.W. Taft, R.M. Doherty, M.J. Kamlet, Chromatographia 25 (1988) 373.
[10] J. Barbosa, R. Bergés, V. Sanz-Nebot, J. Chromatogr. A 719 (1996) 27.
[11] M.J. Kamlet, J.L. Abboud, R.W. Taft, J. Am. Chem. Soc. 99 (1977) 6027.
[12] R.W. Taft, M.J. Kamlet, J. Am. Chem. Soc. 98 (1976) 2886.
[13] M.J. Kamlet, R.W. Taft, J. Am. Chem. Soc. 98 (1976) 377.
[14] W.J. Cheong, P.W. Carr, Anal. Chem. 61 (1989) 1524.
[15] M.J. Kamlet, P.W. Carr, R.W. Taft, M.H. Abraham, J. Am. Chem. Soc. 103 (1981) 6062.
[16] J. Barbosa, V. Sanz-Nebot, I. Toro, J. Chromatogr. A 725 (1996) 249.
[17] D. Barrón, J.A. Pascual, J. Segura, J. Barbosa, Chromatographia 41 (1995) 573.
[18] T.M. Krygowski, P.K. Wrona, U. Zielkowska, C. Reichardt, Tetrahedron 41 (1985) 4519.
[19] Y. Marcus, Y. Migron, J. Phys. Chem. 95 (1991) 400.
[20] C. Reichardt, Solvents and Solvent Effects in Organic Chemistry, VCH, Weinheim, 1988.
[21] J.J. Michels, J.G. Dorsey, J. Chromatogr. 435 (1988) 85.
[22] M.M. Hsieh, J.G. Dorsey, J. Chromatogr. 631 (1993) 63.
[23] C. Molina, M. Honing, D. Barceló, Anal. Chem. 66 (1994) 4444.
[24] H. Gaertner, A. Puigserver, J. Chromatogr. 350 (1988) 279.
[25] C.T. Mant, T.W.L. Burke, J.A. Black, R.S. Hodges, J. Chromatogr. 458 (1988) 205.
[26] C.F. Poole, S.A. Schuette, Contemporary Practice of Chromatography, Elsevier, Amsterdam, New York, 1984.
[27] S. Rondinini, P.R. Mussini, T. Mussini, Pure Appl. Chem. 59 (1987) 1549.
[28] F.G.K. Bauke, R. Naumann, C. Alexander-Weber, Anal. Chem. 65 (1993) 3244.
[29] J. Barbosa, I. Marqués, D. Barrón, V. Sanz-Nebot, Trends Anal. Chem. 18 (1999) 543.
[30] J.G. Dorsey, B.P. Johnson, Chimicaoggi 4 (1986) 23.
[31] P. Roepstorff, J. Fohlman, J. Biomed. Mass Spectrom. 11 (1984) 60.
[32] D. Volmer, K. Levsenn, G. Wünsch, J. Chromatogr. A 660 (1994) 231.