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Separation and characterization of multicomponent peptide mixtures by liquid chromatography–electrospray ionization mass spectrometry
Application to crude products of the synthesis of leuprolide

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Abstract

Leuprolide is a synthetic structural analogue of luteinizing hormone-releasing hormone used for the treatment of a large number of diseases related with the regulation of sexual hormones. Solid-phase peptide synthesis is used to obtain leuprolide peptidic hormone, but this synthetic procedure results in complex mixtures that need separation and characterization. Here, liquid chromatography coupled with mass spectrometry using electrospray ionization, (LC–ES-MS), was used for the separation and characterization of multicomponent peptide mixtures of crudes of synthesis of leuprolide. To optimize the LC separation process, the method of linear solvation energy relationships was applied and the powerful coupling LC–ES-MS permitted rapid and reliable characterization of the reaction product. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Peptides; Hormones; Leuprolide

1. Introduction

The use of peptides in biomedical therapy has increased in the last few years, because they have a large range of activity and specificity, usually with a low toxicity and a rapid metabolization [1,2].

Leuprolide {CAS: [53714-56-0] (leuprolide or leuprorelin) and [74381-53-6] (leuprorelin acetate)} is a synthetic structural analogue of luteinizing hormone-releasing hormone (LHRH), which regulates the production of sexual hormones. Leuprolide presents a higher affinity for the LHRH receptors and a higher resistance to enzymatic degradation than the natural hormone, which increases its biological potential. It can thus be used for the treatment of a large number of diseases related with the regulation of sexual hormones, like: masculine and feminine infertility, uterine myomas and prostatic and mammalian tumors (Fig. 1) [3,4].

The interest in peptidic hormone analogues has led to a parallel development of new strategies within the solid-phase peptide synthesis (SPPS) method. Since Merrifield [5] first introduced this procedure in 1962, it has been used in the synthesis of peptides and small proteins, with new modifications and a large number of improvements. However, this procedure results in complex crudes of reaction, in which the target peptide is mixed with undesired side products, for example incomplete side chains, products resulting from amino acidic insertions and

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Leuprolide

(5-oxo-L-prolyl-L-histidyl-L-tryptophanyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-1-L-arginyl-L-prolyl-ethylamide)

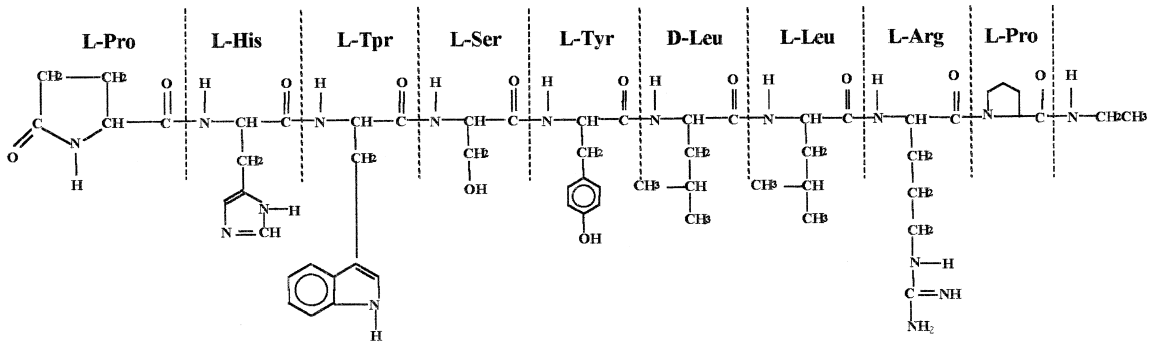


Fig. 1. Structure of leuprolide.

parallel reactions of the side chains of some amino acidic residues, *tert*.-butylated products and racemics [6]. So, the purification of the target peptide and the characterization of the impurities of the crude of synthesis must be solved before the peptidic analogue can be commercialized, in order to obtain a product that fulfils the legal requirements [7]. Therefore, laboratory preparative scale purification of these peptide mixtures needs to be considered. Taking the complexity of crudes of synthesis into account, the preparation of significant amounts of purified peptides requires firstly assessment of specifically analytical scale conditions. Also, purification and characterization allow optimization of the synthetic process, to make it economically beneficial.

The two most commonly used techniques to purify and characterize new synthesis products, liquid chromatography (LC) and capillary electrophoresis (CE) [8–11], lack the mass specificity necessary to allow unambiguous characterization of such complex products. LC and/or CE coupled with mass spectrometry (MS) have proved to be highly efficient for fast and reliable analysis and characterization of crude synthetic products [12–15]. Moreover, in order to achieve a fast optimization of the separation process, the method of linear solvation energy relationships (LSERs) can predict the chromatographic retention [16–19]. In this study, the proportion of the organic modifier of the mobile phase was optimized by establishing relationships between Reichardt's E_T^N scale of solvent polarity and the chromatographic retention measured by the capacity factor, k'. Also characterization of the side products associated with the target peptide leuprolide has been successfully carried out by coupling LC to MS using an electrospray ionization interface (LC–ES-MS). Although new ionization techniques have been developed, electrospray ionisation is the most used in the case of peptides [20–24], due to its sensitivity and its ability to analyze large, thermally labile biomolecules and to provide accurate molecular mass measurements using mass analyzers with limited range such as quadrupoles (m/z 2500).

The powerful coupling LC–ES-MS has become an important aid for rapid and reliable identification of the target peptide and has furnished comprehensive information on other reaction products in the multicomponent peptide mixtures of crude of synthesis of leuprolide peptidic hormone.

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm, acetonitrile (MeCN) (Merck, Darmstadt, Germany) and dioxane (Merck) were of LC grade. Trifluoroacetic acid (TFA), potassium hydrogenphthalate (dried at 110°C before use) were all analytical grade obtained from Merck. The crudes of synthesis of leuprolide (5-oxo–Pro–His–Trp–Ser–Tyr–D-Leu–

Leu–Arg–Pro–NHEt) used in this study was supplied by Lipotec (Barcelona, Spain). Leuprolide crudes were dissolved using the mobile phase as solvent, at concentrations of 1 and 3 mg/ml for LC–UV analysis and LC–ES-MS analysis, respectively, and were stored in a freezer at 0°C when not in use. All the eluents and mobile phases were passed through a 0.22-μm nylon filter (MSI, Westboro, MA, USA) and degassed by bubbling helium. The samples were passed through a 0.45-μm nylon filter (MSI).

2.2. Apparatus

For the LC–UV experiments an ISCO (Lincoln, NE, USA) Model 2350 chromatographic pump with a Valco injection valve with a 20-μl sample loop and a variable-wavelength V^4 absorbance detector (ISCO) operating at 220 nm were used. The chromatographic system was controlled by Chemresearch Chromatographic Data Management System Controller Software (ISCO) running on a personal computer. A 5 μm Kromasil C_8 column (250×4.6 mm I.D.) (BC Aplicaciones Analíticas, Barcelona, Spain) was used at room temperature.

The electromotive force (e.m.f.) values used to calculate the pH of the mobile phase, as in previous works [25], were measured with a potentiometer Model 2002 (Crison Instruments, Barcelona, Spain) using an Orion 8102 Ross Combination pH electrode (Orion Research, Boston, MA, USA) with a precision of ±0.1 mV. The potentiometric system was calibrated using a standard reference solution of potassium hydrogenphthalate 0.05 mol/kg [17] whose reference pH values in the acetonitrile–water mixtures studied were known [26–28].

LC–ES-MS experiments were performed using two Phoenix 20 syringe pumps (CE Instruments, Milan, Italy) with a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 100-μl sample loop, coupled to a VG Platform II quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with a nebulizer-assisted electrospray source. The high-flow nebulizer was operated in a standard mode with N_2 as both nebulizing (15–20 l/h) and drying (400 l/h) gas. Separation was performed at ambient temperature on a 5 μm Kromasil C_8 column (250×4.6 mm I.D.) with 1 ml/min flow-rate. 0.3 ml/min of dioxane were added post-column to improve sensitivity. The total flow was split to allow an effective flow 60 μl/min into the source of the spectrometer. Instrument control and data analysis were performed using Masslynx application software from Micromass. The mass data represent the average of three separate measurements.

2.3. Procedures

2.3.1. LC–UV procedure

For the optimization of the mobile phase composition, the solution used as mobile phase was made of different acetonitrile–water mixtures containing 0.1% (v/v) TFA (pH 1.9–2.0) [16], at several concentrations of acetonitrile from 25% to 35% (v/v). The flow-rate of mobile phase was 1 ml/min. Capacity factors were calculated from $k' = (t_R - t_0)/t_0$, where t_0 is the hold-up time, established for every mobile phase composition using 0.01% (w/v) bromide solution in water [29], and t_R is the retention time of the target peptide and the associated products. The retention times and the capacity factors of the solutes were determined from three injections of 1 mg/ml solution of leuprolide crude at each mobile phase composition considered and monitoring the signal at 220 nm. The pH was measured in the mixed mobile phase in which the chromatographic separation took place [30].

2.3.2. LC–MS procedure

2.3.2.1. Optimization of the source and analyzer parameters

The source and analyzer parameters were optimized using electrospray ionization of 0.1 mg/ml crude solution in MeCN–water (31:69), 0.1% (v/v) TFA, introduced directly into the ES source. Parameters were optimized in order to obtain the best signal, stability of the measurement and the highest sensitivity of the target peptide leuprolide. Optimum conditions were as follows: flow-rate, 60 μl/min; capillary voltage 4000 V; counter electrode voltage, 250 V; sample cone voltage, 70 V; source temperature, 90°C; ion energy, 3 V. Fragmentation was also studied under these working conditions. At lower sample cone voltages ES yields simple mass spectra

with negligible fragmentation, allowing correct identification of the molecular identities. For this reason, spectra were obtained using extraction voltages of 40 V, as far as possible. At percentages of acetonitrile of 25 to 29%, extraction voltages of 70 V were used owing to the analyte signal reduction observed at lower acetonitrile compositions, probably due to the higher surface tension of the solution, which results in an inefficient spray process [31].

2.3.2.2. Identification of side products

For the identification of the side products of the crude of synthesis, 3 mg/ml solution of leuprolide crude was injected into the LC–ES-MS system, using acetonitrile–water containing 0.1% (v/v) TFA (28:72 and 33:67, v/v) as mobile phase. The pH measured in these mixtures ranged between 1.9 and 2. ES spectra of target peptide leuprolide and associated side products were obtained at the optimum conditions of the mass spectrometer and working at full scan mode (m/z 400–1500).

3. Results and discussion

3.1. LC–UV

The synthesis crude of leuprolide was first examined by analytical LC with UV detection. The resulting chromatograms contain a major peak corresponding to the target peptide leuprolide, as well as a number of peaks of unidentified peptidic substances (I1, I2, I3, I4, I5 and I6) (Fig. 2). To optimize the composition of mobile phase, the LSER method, which is based on the Kamlet–Taft multiparameter scale, was used in the same way as in previous works [16–19]. The LSER approach applied to chromatographic processes [32–35] correlates retention parameters of solutes with characteristic properties of solutes and both stationary and mobile phases. This correlation can be expressed as follows in a system with a fixed pair of solute and stationary phase [16,17]:

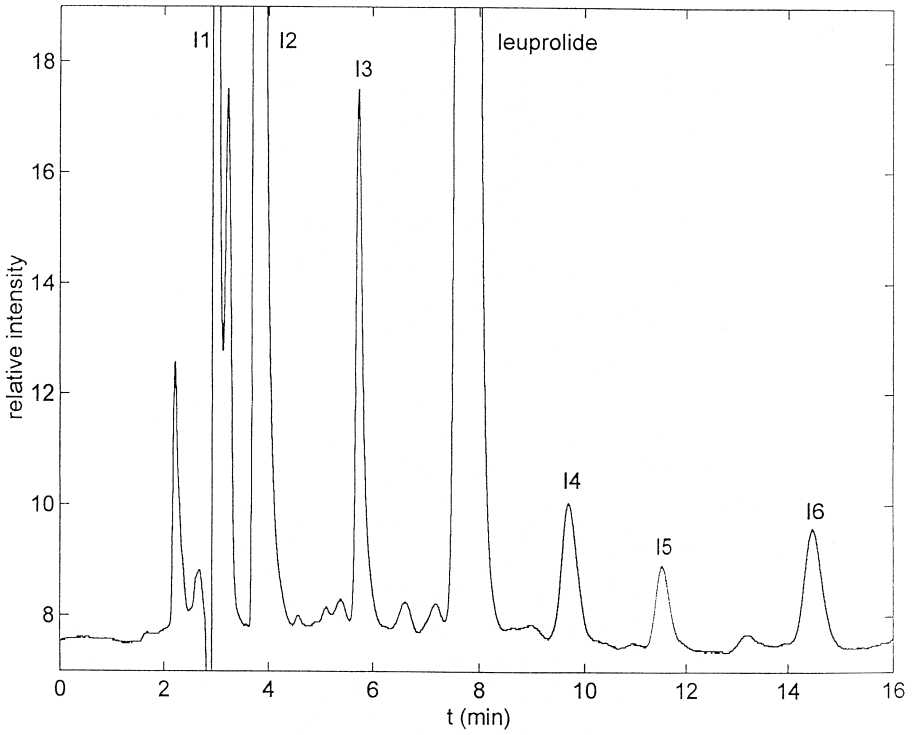


Fig. 2. UV chromatogram of a solution containing 1 mg/ml of a crude of synthesis of leuprolide. Mobile phase: MeCN–water (31:69), 0.1% (v/v) TFA.

$$\log k' = (\log k')_0 + s\pi^* + a\alpha + b\beta \quad (1)$$

where the coefficients $(\log k')_0$, s, a and b depend on the solute and the stationary phase properties and π^*, α and β are the solvatochromic parameters used to evaluate solvent dipolarity/polarizability [36], hydrogen-bond acidity [37] and hydrogen-bond basicity [38], respectively. Taking into account that the values of β are constant over most of the acetonitrile composition range [39,40] and the parameters E_T^N [41], π^* and α correlate according to $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [42], Eq. (1) can be reduced to Eq. (2) as follows:

$$\log k' = c + eE_T^N \quad (2)$$

This relation has been applied in previous studies for predicting the retention of analytes with very different structural characteristics [16–19]. In this work, k' values of leuprolide and associated side products, were obtained using acetonitrile–water systems as mobile phases, with acetonitrile percentages from 25% to 35% (v/v) and are shown in Table 1. The values of log k' were plotted versus the E_T^N values of the mixtures studied in Fig. 3, where a good linearity, greater than 0.99, was observed. This linearity allows prediction of the elution behavior of peptide derivatives and hence optimization of eluent composition for best separation from only two experimental measurements of k' values [16–19].

In order to examine the accuracy of retention prediction by Eq. (2), the selectivity for adjacent solute pairs calculated in the usual way, $\alpha = k_i'/k_j'$, was employed. Theoretical selectivity values were obtained using predicted k', calculated through Eq. (2) from only two experimental points, corresponding to the lowest and the highest acetonitrile percentages of the interval studied. Fig. 4 plots experimental α values as points, calculated using experimental k' values given in Table 1, and theoretical α values as solid lines vs. the acetonitrile percentage, showing a good concordance of both selectivity values. For our particular case, the composition interval between 28% and 32% of acetonitrile provided the best ratio selectivity/analysis time: working under 28% of acetonitrile the analysis times were too large, and over 32% of acetonitrile selectivity between impurity 3 and leuprolide was poor.

Moreover, a good resolution between all the analytes of interest in the leuprolide crude was our main goal. Fig. 5 shows variation of resolution (R_s) for the solute pairs versus percentage of acetonitrile in the mobile phase. Solid lines indicate theoretical resolution values obtained using Eq. (3):

$$R_s(\text{theo}) = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2'}{1 + k_2'}\right) \quad (3)$$

where N is the number of theoretical plates [$N = 16(t_R/w)^2$], α the theoretical selectivity and k' the capacity factors calculated using Eq. (2). The points indicate experimental resolution values obtained from the typical relation: $R_s(\text{exp}) = (t_{R2} - t_{R1})/(w_2 - w_1)$.

Only resolution between solutes whose separation was critical was considered. The concordance of the theoretical and experimental R_s values in Fig. 5 confirms accurate estimations of resolution via Eq.

Table 1
Capacity factor values of the leuprolide and the associated products, and the E_T^N parameter values, at the percentages of acetonitrile assayed in the mobile phase

% (v/v) MeCN	E_T^N	k'(I1)	k'(I2)	k'(I3)	k'(leuprolide)	k'(I4)	k'(I5)	k'(I6)
25	0.877	0.360	0.878	2.21	14.84	22.70	28.61	33.33
26	0.873	0.339	0.816	2.04	10.57	15.85	19.74	23.74
27	0.869	0.337	0.778	1.93	7.60	11.10	13.88	17.03
28	0.865	0.330	0.750	1.84	5.87	8.42	10.48	13.12
29	0.862	0.318	0.711	1.72	4.357	6.10	7.59	9.64
30	0.859	0.310	0.665	1.61	3.067	4.24	5.30	6.91
31	0.855	0.300	0.631	1.51	2.332	3.24	4.03	5.33
32	0.852	0.295	0.616	1.44	1.860	2.55	3.16	4.24
33	0.849	0.299	0.599	1.38	1.492	2.02	2.52	3.39
34	0.847	0.282	0.572	–	1.161	1.56	1.94	2.65
35	0.844	0.281	0.562	–	0.920	1.23	1.54	2.09

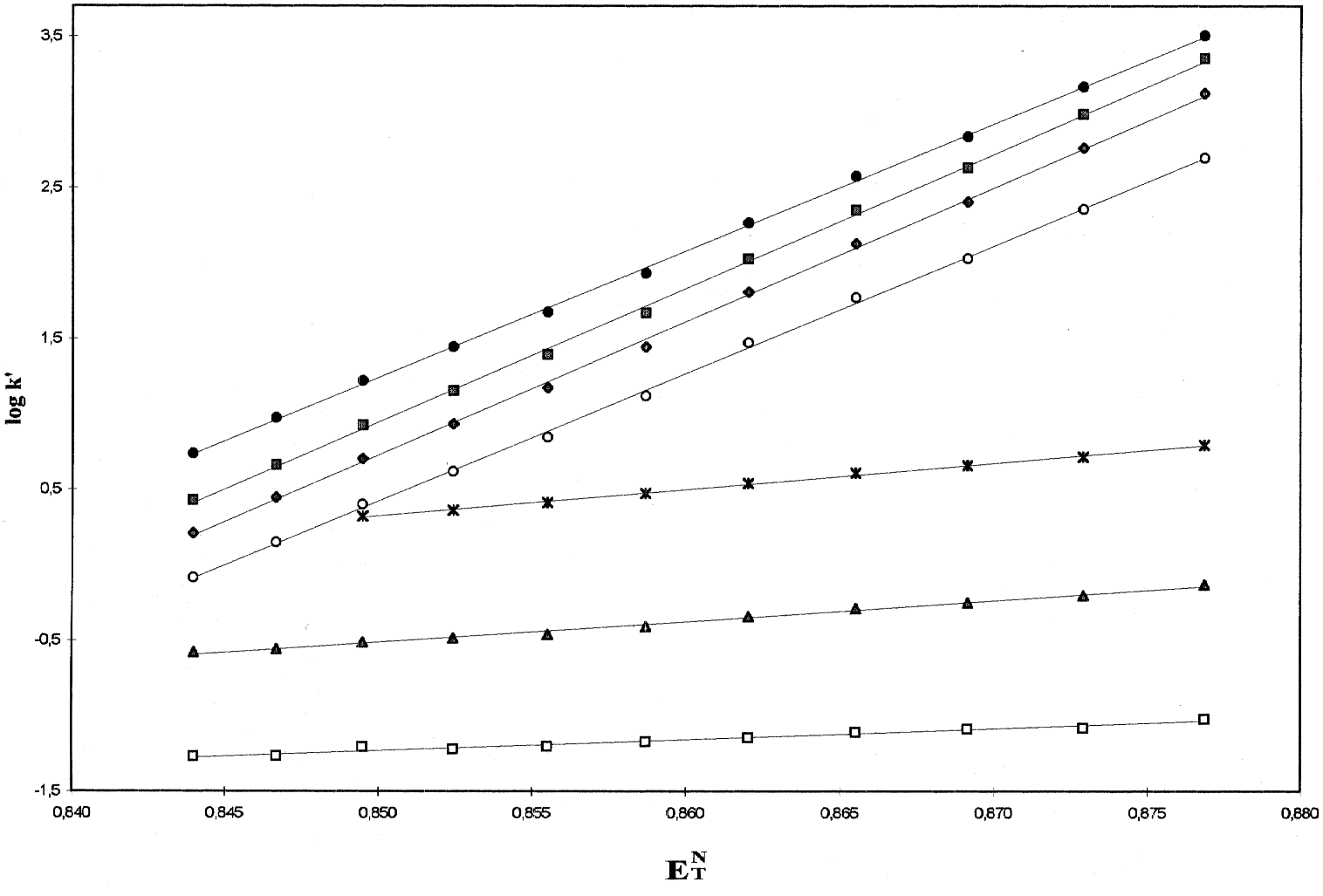


Fig. 3. Plots of log k' of leuprolide and impurities versus E_T^N parameters of the mobile phase: impurity I1 (□), impurity I2 (▲), impurity I3 (∗), leuprolide (○), impurity I4 (♦), impurity I5 (■) and impurity I6 (●).

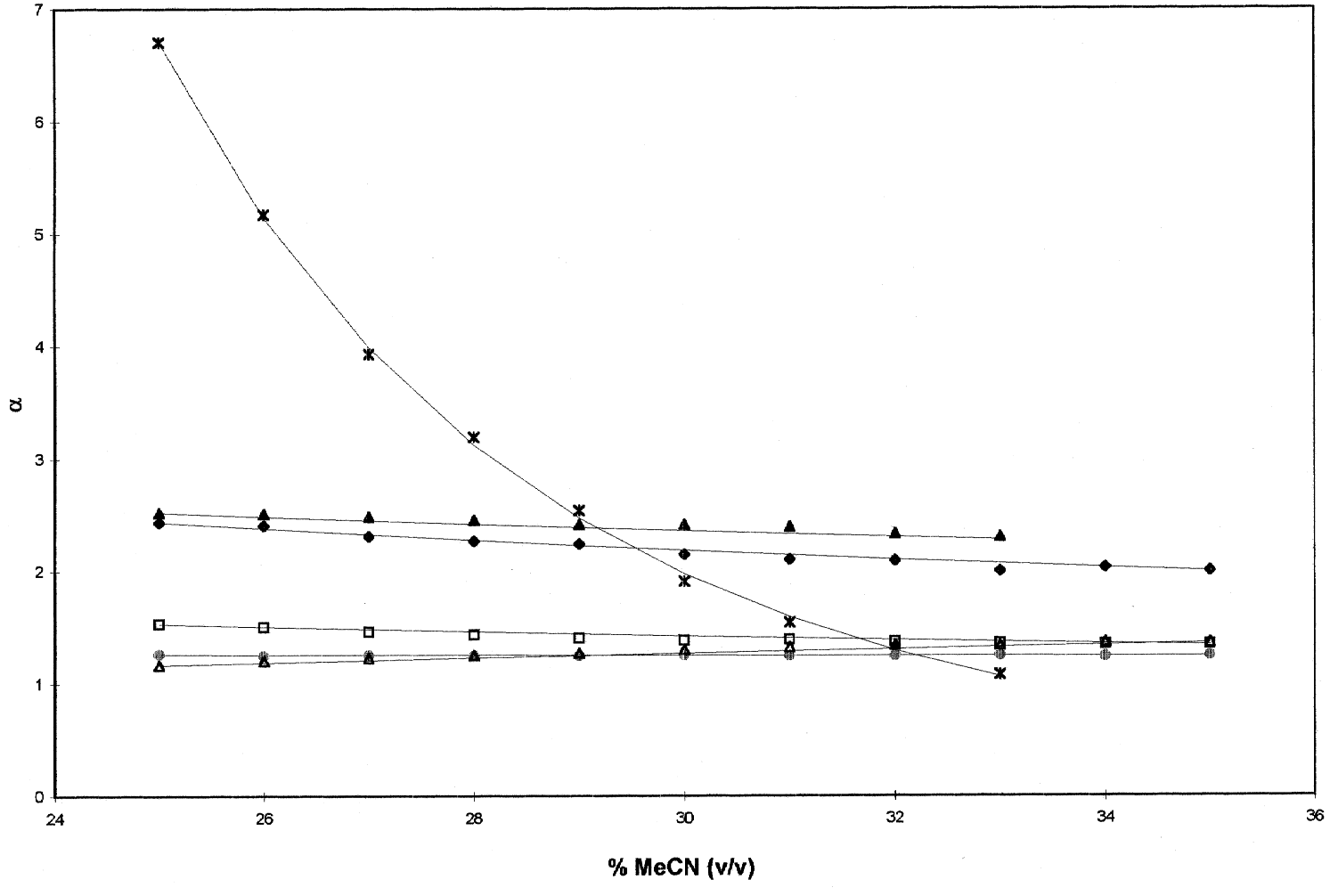


Fig. 4. Plots of selectivity between adjacent pairs of solutes versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of selectivity and points indicate experimental values of selectivity: impurity I1/impurity I2 (♦), impurity I2/impurity I3 (▲), impurity I3/leuprolide (∗), leuprolide/impurity I4 (□), impurity I4/impurity I5 (●) and impurity I5/impurity I6 (△).

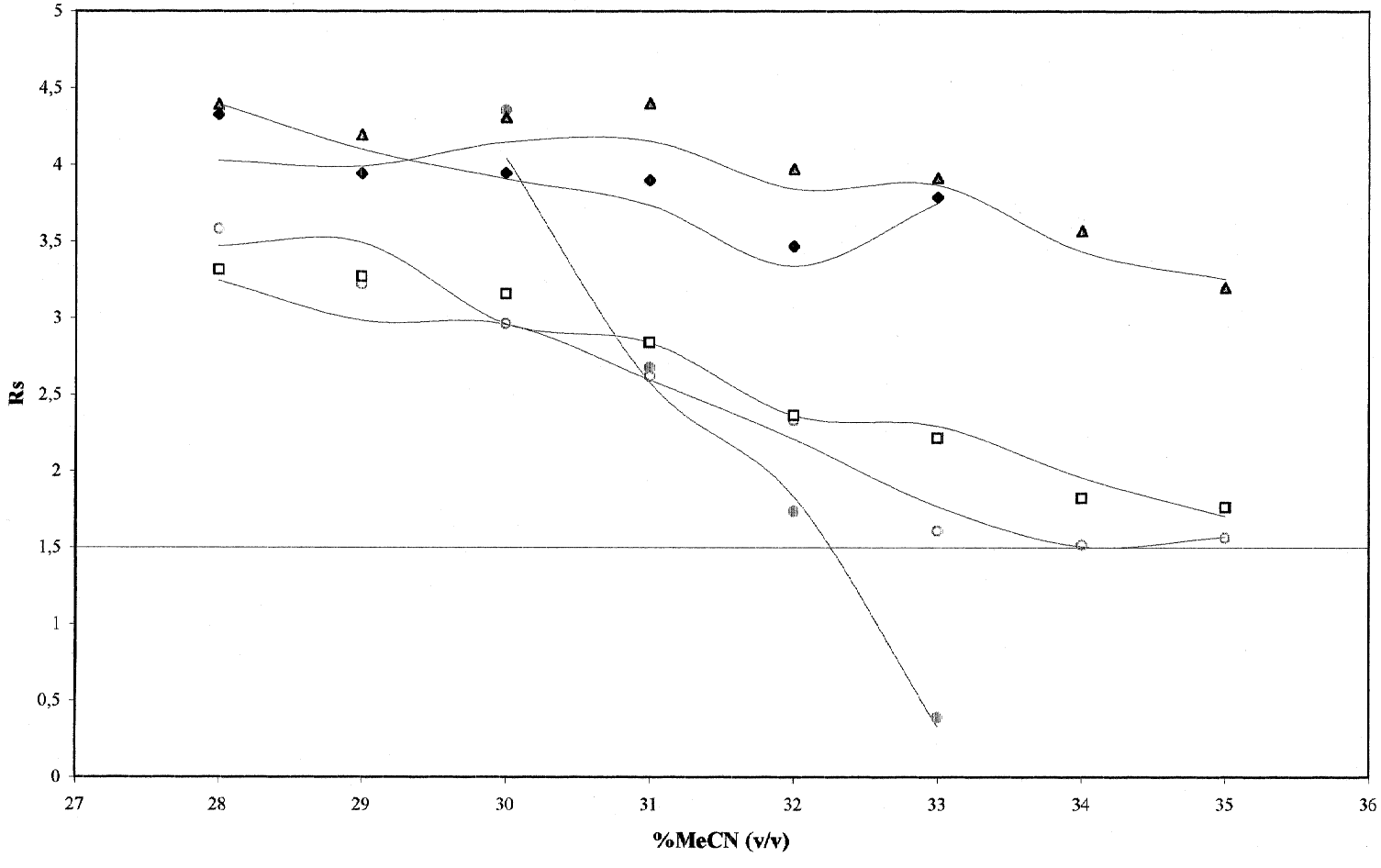


Fig. 5. Plots of resolution between adjacent pairs of solutes versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of resolution from Eq. (3) and points represent experimental values of resolution: impurity I2/impurity I3 (♦), impurity I3/leuprolide (●), leuprolide/impurity I4 (○), impurity I4/impurity I5 (□) and impurity I5/impurity I6 (▲).

(2) from only two experimental measurements. From Figs. 3, 4 and 5 we conclude that the optimum separation between the target peptide and the side products present in the crude could be achieved at percentages of acetonitrile in the mobile phase between 28 and 32% (v/v). The chromatogram of the crude of synthesis of leuprolide using UV detection obtained from injection of 1 mg/ml solution at optimal conditions is shown in Fig. 2. On the other hand, the mobile phase recommended for the purification of leuprolide using preparative chromatography is 31% (v/v) acetonitrile in water, 0.1% (v/v) TFA; this eluent provides high resolutions ($R_s \cong 3$) and suitable analysis times.

3.2. LC–MS

LC–MS was performed under the conditions described in the experimental section. In order to improve the analytical signal obtained, post-column additions of several solvents (acetonitrile, propionitrile, ethanol, methanol, dioxane and tetrahydrofuran) were made [31,43]. The addition of 0.3 ml/min of dioxane provided a signal enhancement (Fig. 6), whereas no net improvement was observed with the other solvents.

In order to study the synthetic subproducts that eluted near to leuprolide, a mobile phase with an acetonitrile percentage of 28% (v/v) was chosen (Fig. 7a). On the other hand, working with richer acetonitrile mobile phases (Fig. 7b), additional impurities (I7 to I15) were observed. These side products did not interfere in the leuprolide purification because of their different retention times, but their characterization could be very useful for the later synthetic process optimization. Comparing the resulting total ion current (TIC) chromatogram of Fig. 7a with the UV chromatogram of Fig. 2, very different intensities for some impurities as I1, I2 and I3 can be observed. They could be non-peptidic substances involved in the synthesis such as solvents and auxiliary reagents, with chromophor groups, which show good UV detection but they can not be observed using mass spectrometry. This is in good agreement with the different slopes observed in the plot log k' vs. E_T^N for these compounds with respect to the peptidic impurities (Fig. 3). On the other hand, the peaks A–E in Fig. 7a and Fig. 7b, are side products detected by mass spectrometry but not assigned in the UV chromatogram.

All the spectra associated with the chromatographic peaks observed in the TICs are shown in Figs. 8 and 9. The mass-to-charge ratios observed, the respective charged states and the molecular masses measured for each substance are summarized in Table 2, as well as the assignment of each ion on each peptide. The molecular masses were calculated from both the singly and doubly charged present in the spectra [44]. It is important to consider the method of the peptide synthesis and the reagents added in each step of the synthesis procedure in order to identify the target peptide and most associated impurities [45]. Leuprolide was prepared by Lipotec applying a linear SPPS method previously developed and optimized, which consists in coupling successively each amino acid of the sequence until it has been completed. After the last coupling, peptide was cleaved from the resin and deprotected by acidolytic treatment, and amidated to obtain the final product leuprolide, Fig. 1. In order to avoid consecutive additions [46] in a coupling step, protection of the α-amino group was carried out using the group 9-fluorenylmethoxycarbonyl (Fmoc). In the same way, reactivity of side chains was avoided by protection with 2,2,5,7,8-pentamethylcroman-6-sulfonyl (Pmc) to block the α-amino group of proline, *tert*.-butyl groups (^{t}Bu) for the hydroxyls of the side chains of tyrosine and serine and triphenylmethyl group (Trt) to block the tertiary amino group of the imidazole ring of tryptophan [6].

3.2.1. Target peptide

The electrospray spectra of the target peptide leuprolide, at retention time $t_R = 13.14$ min (28% MeCN) and 5.82 min (33% MeCN), are shown in Fig. 8e and Fig. 9a, respectively. The two charge states observed are: m/z 1210.0 $[L+H]^+$ and m/z 605.8 $[L+2H]^{2+}$. These ions are associated with the molecular mass 1209.3 which is in good agreement with the expected molecular mass 1209.4. No significant fragmentation is observed at a cone sample voltage of 40 V, but using 70 V the fragmentation present does not difficult this unambiguous assignment of the molecular peak with one and two charges.

The spectra of the side products identified in the

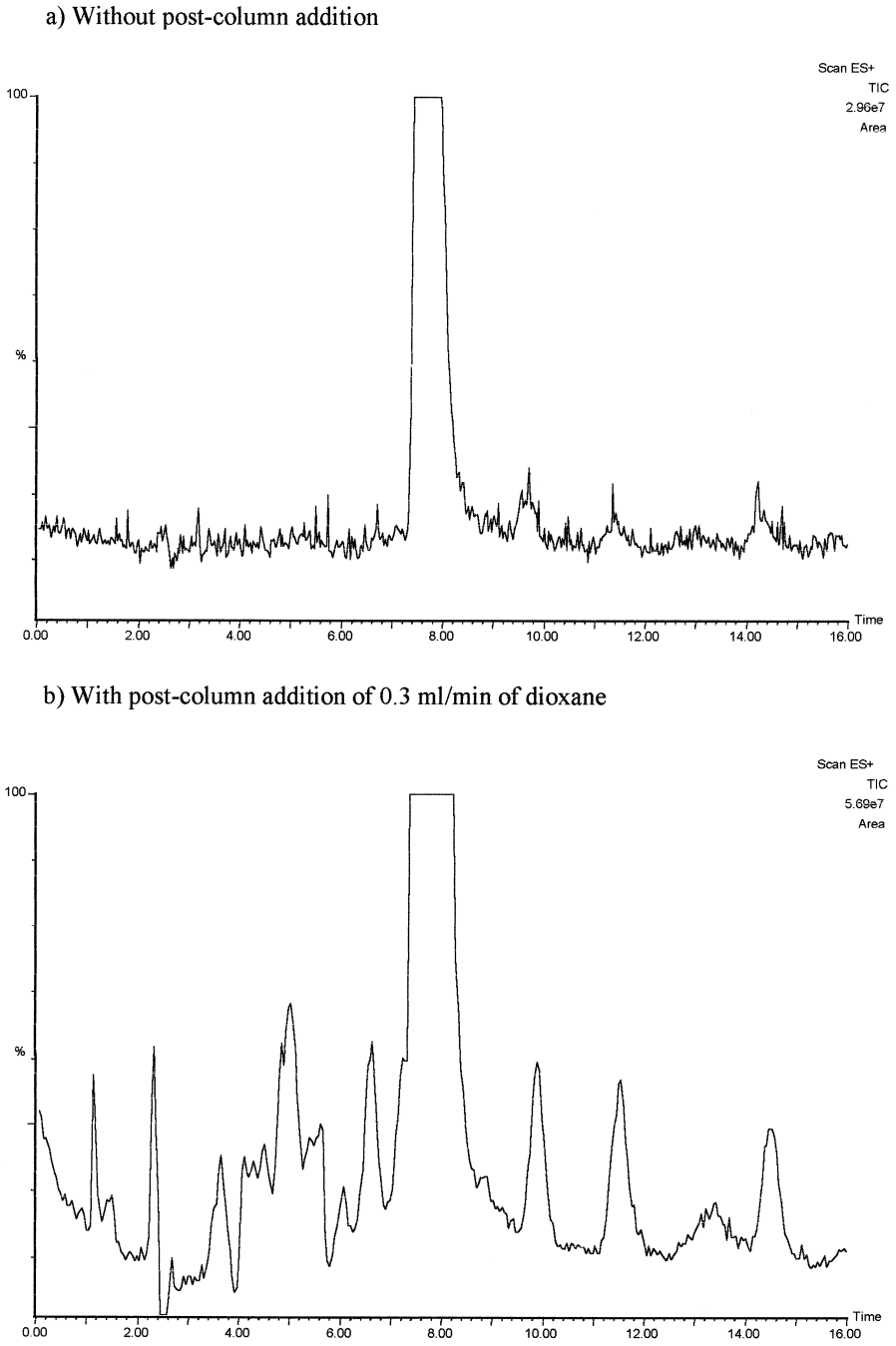


Fig. 6. Total ion current (TIC) chromatogram of a 3 mg/ml solution of leuprolide crude, with a mobile phase of MeCN–water (31:69), 0.1% (v/v) TFA. (a) Without post-column addition, (b) with post-column addition of 0.3 ml/min of dioxane. Time scales in min.

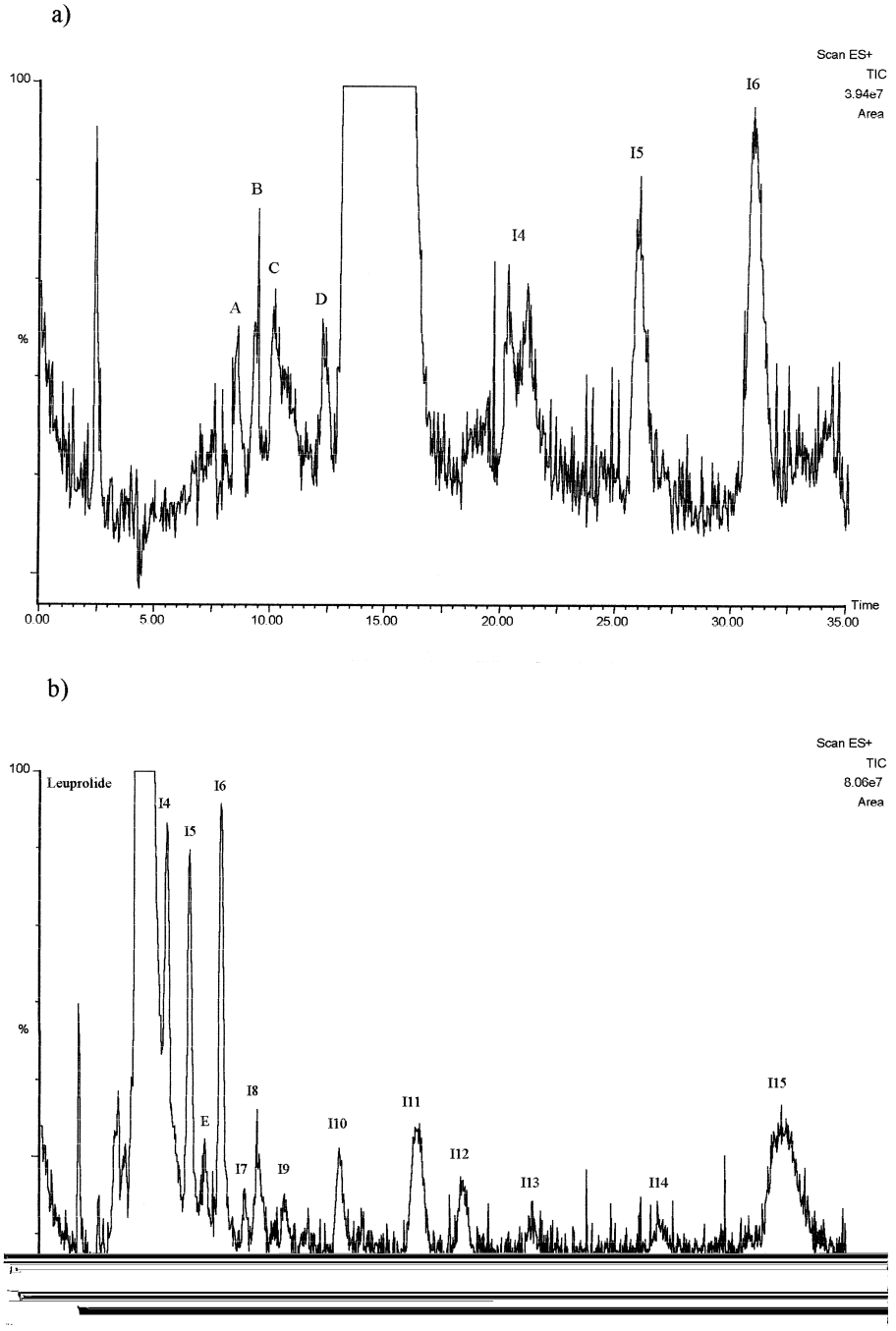


Fig. 7. (a) Total ion current (TIC) chromatogram of a 3 mg/ml solution of leuprolide crude, with a mobile phase of MeCN–water (28:72), 0.1% (v/v) TFA. (b) TIC chromatogram of a 3 mg/ml solution of leuprolide crude, with a mobile phase of MeCN–water (33:67), 0.1% (v/v) TFA. Time scales in min.

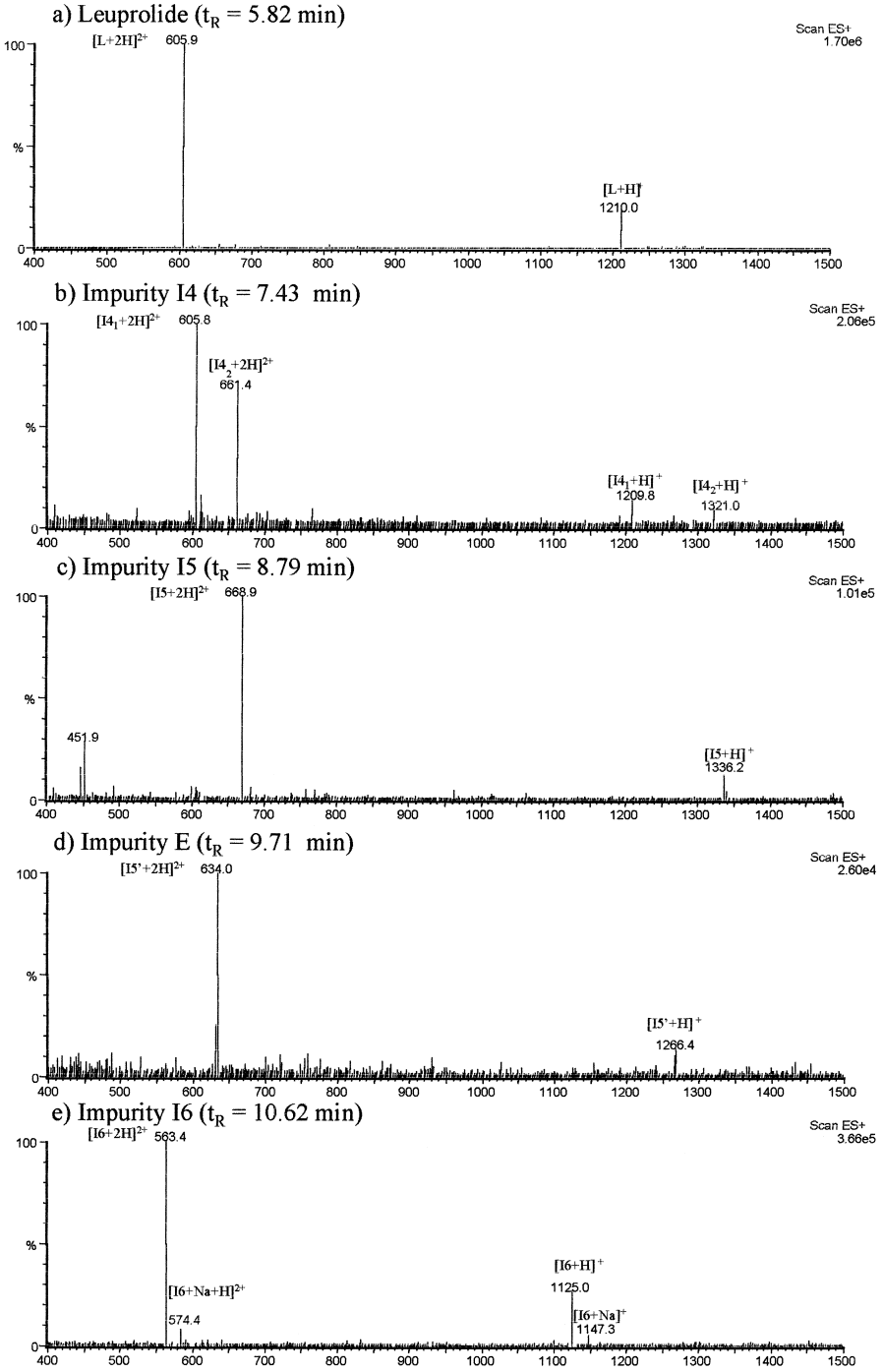


Fig. 9. Electrospray mass spectra associated with the TIC peaks at the retention time indicated in each case. Sample cone voltage: 40 V, mobile phase: MeCN–water (33:67), 0.1% (v/v) TFA. *x*-Axes: *m*/*z*.

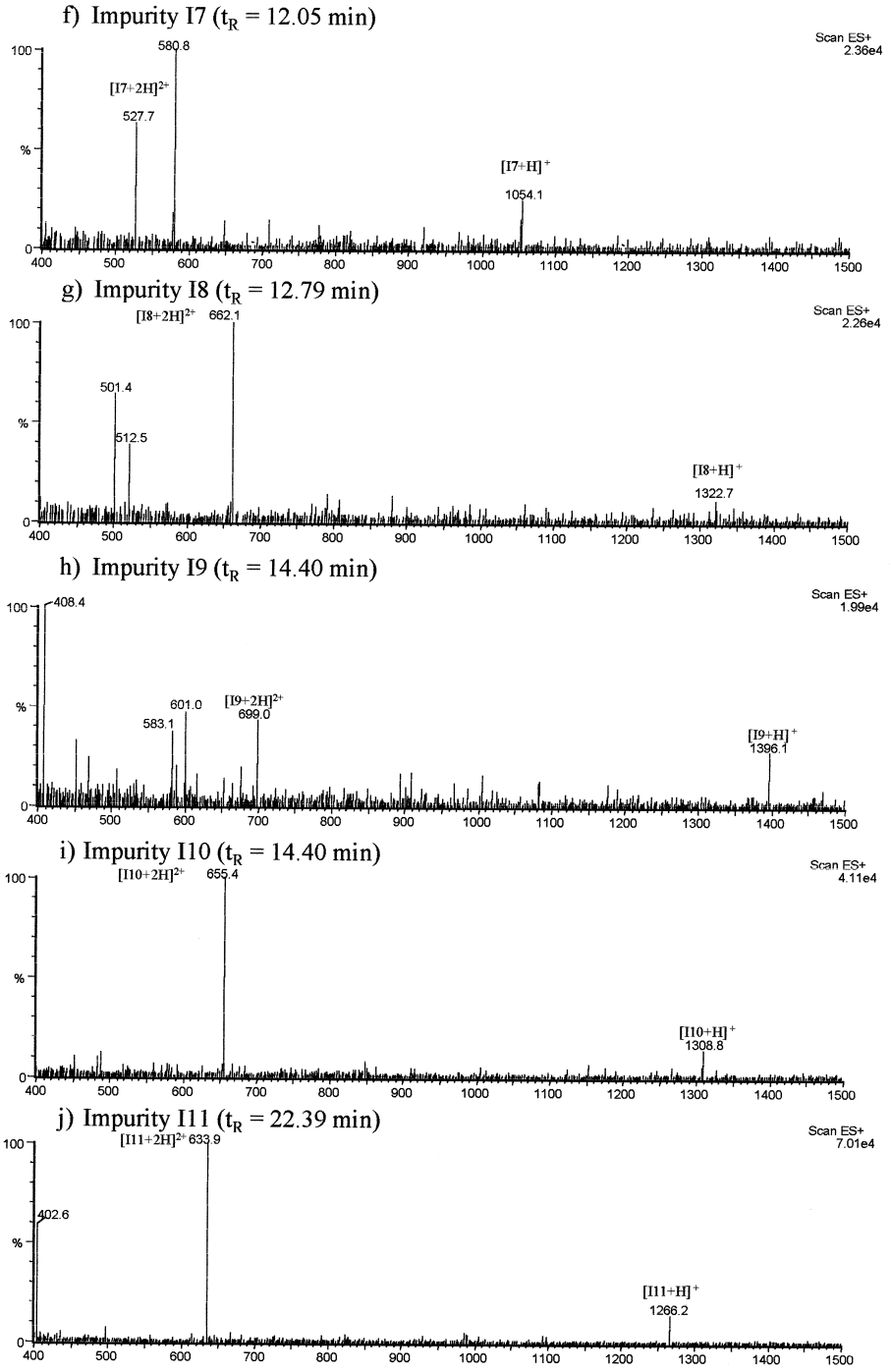


Fig. 9 (*continued*).

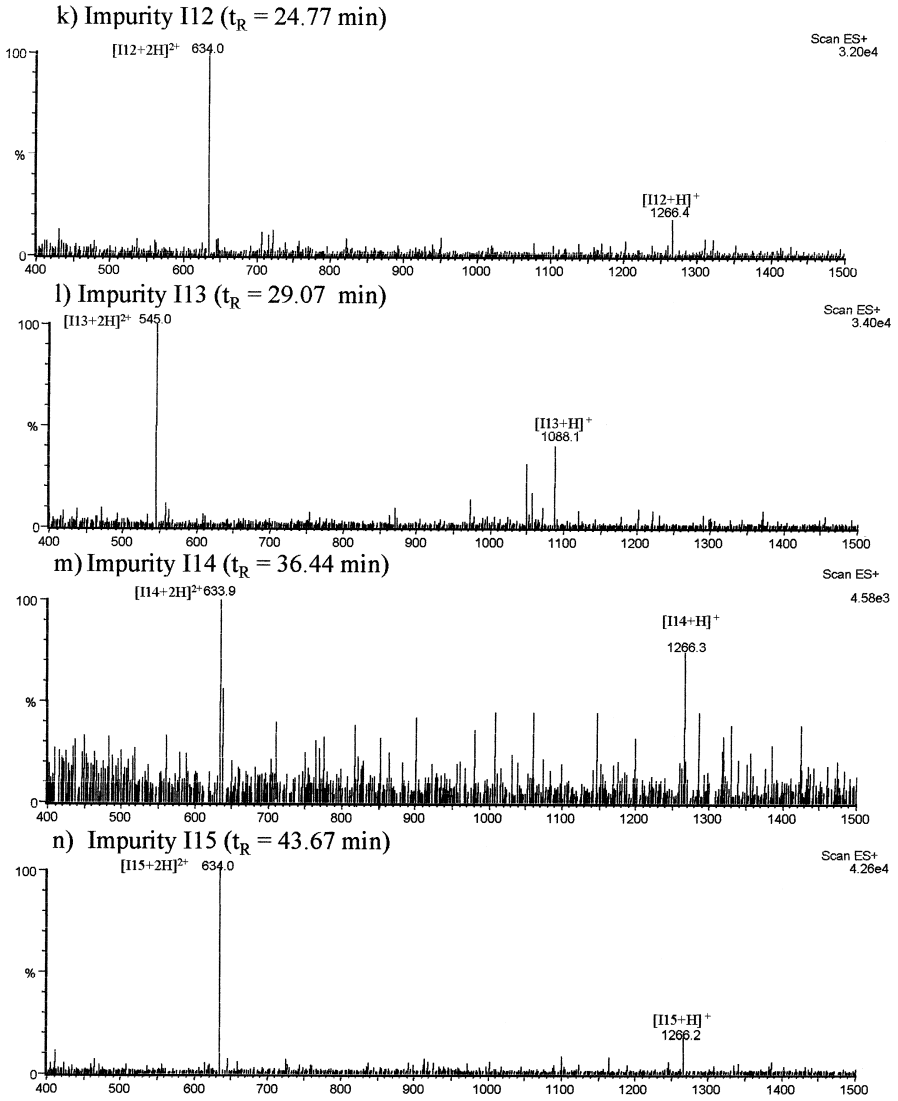


Fig. 9 (*continued*).

synthesis crude are shown in Figs. 8 and 9 and their identification is based on the experimental mass differences between the expected molecular mass of leuprolide, 1209.4, and the observed molecular mass of the side product [47].

3.2.2. Amino acidic insertions

There are some impurities in the crude of leuprolide, attributed to the insertion of an additional amino acid in the sequence while coupling step was carried out. The major reason for this occurrence in solid-phase peptide chemistry is the use of an excess of equivalents in the coupling step to assure the maximum coupling efficiency, taking into account the low coupling rates of some amino acids [46]. The spectrum corresponding to the chromatographic peak I9 ($t_R = 14.4$) (Fig. 9h) presents a double-charge state distribution: the m/z 1396.1 $[I9+H]^+$ and the m/z 699.0 $[I9+2H]^{2+}$, that yield the molecular mass 1395.5, 186.1 higher than the leuprolide molecular mass. This difference agrees with the insertion of an additional tryptophan in the sequence of leuprolide:

Table 2
Measured relative molecular masses (M_r) and proposed sequences associated with the TIC peaks in Fig. 7

Impurity	t_R (min)		Observed m/z[a]	Measured M_r	ΔM	Proposed sequence
	28% ACN	33% ACN				
			$[M+2H]^{2+}$ $[M+H]^+$			
A	8.80	–	606.6 1211.9	1211.1	+1.7	Leuprolide with a reduced tryptophan
B1	9.68	–	606.6 1211.8	1211.1	+1.7	Leuprolide with a reduced tryptophan
B2	9.68	–	683.7 1366.5	1365.5	+156.1	Additional arginine insertion
B3	9.68	–	613.5 1225.9	1225.0	+15.6	Leuprolide with an oxidized tryptophan
C	10.73	–	674.2 1346.9	1346.1	+136.7	Additional histidine insertion
Leuprolide	13.14	5.82	605.8 1210.0	1209.3	0	Leuprolide
$I4_1$	–	7.43	605.8 1209.8	1209.2	−0.2	Leuprolide, racemic
$I4_2$	–	7.43	661.4 1321.0	1320.4	+111.0	Fmoc–Hys–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–CO–$NHCH_2CH_3$
I5	–	8.79	668.9 1336.2	1335.5	+126.1	Formation of a substituted guanidine
E	–	9.71	634.0 1266.4	1265.7	+56.3	*tert*.-Butylated leuprolide
I6	–	10.62	563.4 1125.0 $[M+H+Na]^{2+}$ $[M+Na]^+$ 574.3 1147.2	1224.4	−85.0	Lack of a serine
I7	–	12.05	527.8 1054.0	1053.3	−156.1	Lack of an arginine
I8	–	12.79	662.1 1322.7	1321.9	+112.5	Doubly *tert*.-butylated leuprolide
I9	–	14.4	699.0 1396.1	1395.5	+186.1	Additional tryptophan insertion
I11	–	22.39	633.9 1266.2	1265.5	+56.1	*tert*.-Butylated leuprolide
I12	–	24.77	634.0 1266.4	1265.7	+56.3	*tert*.-Butylated leuprolide
I14	–	36.44	633.9 1266.3	1265.5	+56.1	*tert*.-Butylated leuprolide
I15	–	43.67	634.0 1266.2	1265.6	+56.2	*tert*.-Butylated leuprolide

Not identified: D, I10, I13

[a] Observed m/z represent the mean of three LC–ES-MS analyses.

5-oxo-Pro-His-Trp-Trp-Ser-Tyr-D-Leu-L-Leu-Arg-Pro-CO-NHCH$_2$CH$_3$ Impurity I9

The charge-to-mass ratios presents in the spectrum of peak B (t_R=9.68) (Fig. 8b): m/z 1366.5 $[B2+H]^+$ and m/z 683.7 $[B2+2H]^+$, show that one of the impurities that coelutes in this peak has a molecular mass of 1365.5 (difference of mass respect to leuprolide $\Delta M=+156.1$) attributed to the presence of an additional arginine in the sequence of leuprolide:

5-oxo-Pro-His-Trp-Ser-Tyr-D-Leu-L-Leu-Arg-Arg-Pro-CO-NHCH$_2$CH$_3$ Impurity B2

In the case of the chromatographic peak C (t_R=10.73) (Fig. 8c) the charge-to-mass ratios: m/z 1346.9 $[C+H]^+$ and m/z 674.2 $[C+2H]^+$ are present, yielding a molecular mass of 1346.1 (leuprolide $\Delta M=+136.7$) that implicates the presence of an additional histidine in the leuprolide sequence:

5-oxo-Pro-His-His-Trp-Ser-Tyr-D-Leu-L-Leu-Arg-Pro-CO-NHCH$_2$CH$_3$ Impurity C

3.2.3. Incomplete deprotection: protected peptides, deletion peptides and truncated sequences

3.2.3.1. Protected peptides

The presence of impurities with a molecular mass that exceeds 56 the molecular mass of leuprolide is frequent in this peptidic mixture. This has been attributed to the presence in the sequence of a *tert*.-butylated amino acid, as a result of a failed deprotection of the side chains blocked with the *tert*.-butyl group during the chain elongation [6,46]. The spectra associated with the chromatographic peaks E (t_R=9.71), I11 (t_R=22.39), I12 (t_R=24.77), I14 (t_R=36.44) and I15 (t_R=43.67) show similar profiles as can be seen in Fig. 9d, j, k, m and n, respectively: they contain two charge state ratios corresponding to about the values of: m/z 1266 $[Ii+H]^+$ and m/z 634 $[Ii+2H]^{2+}$, yielding measured molecular masses between 1265.5 and 1265.7, that differ from the leuprolide mass by approximately +56. There are two possible *tert*.-butylation places: the hydroxyl groups of the side chains of serine and tyrosine, respectively, but also the indole ring of the side chain of tryptophan could undergo *tert*.-butylation. We also found in the mixture studied the impurity corresponding to the doubly terbutylated leuprolide: the spectrum of the chromatographic peak I8 (t_R=12.79) (Fig. 9g) shows a charge state distribution with the m/z 1322.7 $[I8+H]^+$ and the m/z 662.1 $[I8+2H]^{2+}$; these ions are associated with the molecular mass 1321.9, and the difference of 112.5 with the molecular mass of leuprolide indicates the presence of two *tert*.-butyl groups in the molecule.

3.2.3.2. Truncated sequences

Problems during the deprotection process of the g-fluorenylmethoxycarbonyl (Fmoc) blocking groups could originate incomplete protected sequences [6,46].The spectrum of the peak I4 (t_R=7.43) (Fig. 9b) shows the coelution of two substances. The charge states m/z 1321.0 $[I4_2+H]^+$ and m/z 661.5 $[I4_2+2H]^{2+}$, correspond to the minor substance, yielding the molecular mass of 1320.4 (difference of mass with leuprolide, $\Delta M=+111.0$). This mass is attributed to this sequence:

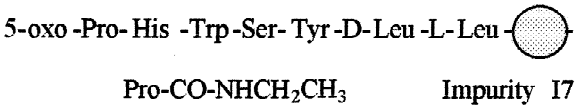
Fmoc–His–Trp–Ser–Tyr–D-Leu–Leu–Arg–Pro–NH–CH$_2$CH$_3$ impurity I4$_2$

3.2.3.3. Deletion peptides

Imperfections in the removal of blocking groups can also lead to the formation of chains from which one of the amino acid residues is absent. Such materials were designated ‘‘failure sequences’’ or ‘‘deletion sequences’’ [47]. The spectrum of peak I6 (t_R=10.62) (Fig. 9e) shows four charge states, corresponding to: m/z 1125.0 $[I6+H]^+$, m/z 563.4 $[I6+2H]^{2+}$ and m/z 1147.2 $[I6+Na]^+$, m/z 574.3 $[I6+Na+H]^{2+}$, yielding a molecular mass of 1124.4 ($\Delta M=-85.0$), which implies the loss of a serine residue:

5-oxo-Pro-His-Trp-Tyr-D-Leu-L-Leu-Arg-Pro-CO-NHCH$_2$CH$_3$ Impurity I6

In the case of peak I7 ($t_R = 12.05$)) (Fig. 9f) the spectrum shows two charge states: m/z 1054.0 $[I7+H]^+$, m/z 527.8 $[I7+2H]^{2+}$, that means that the impurity has a molecular mass of 1053.3 (difference of mass compared with leuprolide $\Delta M = -156.1$), associated to a side product with an arginine deletion in the leuprolide sequence:



3.2.4. Side chain reactivity

The side chain of tryptophan is quite reactive in acidic conditions, and it can undergo oxidations, dimerizations or reductions [48]. In the case of chromatographic peak B ($t_R = 9.68$) (Fig. 8b) one of the products which coelute presents the charge states m/z 1225.9 $[B3+H]^+$ and m/z 613.5 $[B3+2H]^{2+}$, corresponding to a molecular mass of 1225.0, attributed to the oxidation of the indole ring of tryptophan, because the difference of mass compared with leuprolide is 15.6. But the reaction conditions change throughout the synthesis process, so the spectrum associated with peak A ($t_R = 8.8$) (Fig. 8a) shows two charge states, m/z 1211.9 $[A+H]^+$ and m/z 606.6 $[A+2H]^{2+}$, associated with the molecular mass 1211.1 ($\Delta M = +1.7$) that could be tentatively considered as an impurity with the same sequence of leuprolide but with the indole ring of tryptophan reduced. This reduction could be attributed to the presence of triisopropylsilane during the cleavage of the peptide–resin bond [49]. In the same way, one of the impurities that coelutes in chromatographic peak B (Fig. 8b) shows the same charge distribution (m/z 1211.8 $[B1+H]^+$ and m/z 606.6 $[B1+2H]^{2+}$) and it has been associated to a racemic of impurity A.

Histidine also shows reactivity in its side chain. The spectrum associated with peak I5 ($t_R = 8.79$) (Fig. 9c), shows the charge-to-mass ratios m/z 1336.2 $[I5+H]^+$ and m/z 668.9 $[I5+2H]^{2+}$. These ions are associated to the molecular mass 1335.5 ($\Delta M = +126.2$), attributed to the formation of a substituted guanidine by the reaction of the imidazol ring of the histidine side chain when a carbodiimide is added in the coupling step (Fig. 10) [46].

3.2.5. Racemization products

Among the undesired reactions that accompany various operations of peptide synthesis, racemization is the most general cause for concern, occurring in several steps of the synthesis process, for example during the activation and coupling or during the deprotection step and cleavage from the resin, influencing a lot of factors difficult to control [50]. High concentration of reactives are usually used in the coupling steps to secure high coupling rates, minimizing in this way the racemization; the use of additives like 4-dimethylaminopyridine (4-DMAP) or 1-hydroxybenzotriazole (HOBt) with the same purpose is also extended [51]. A racemic of leuprolide has been identified in I4 ($t_R = 7.43$) (Fig. 9b), which shows the charge states: m/z 1209.8 $[I4_1+H]^+$ and m/z 605.8 $[I4_1+2H]^{2+}$, yielding a measured molecular mass of 1209.2, that differs by 0.2 with respect to the expected mass of leuprolide.

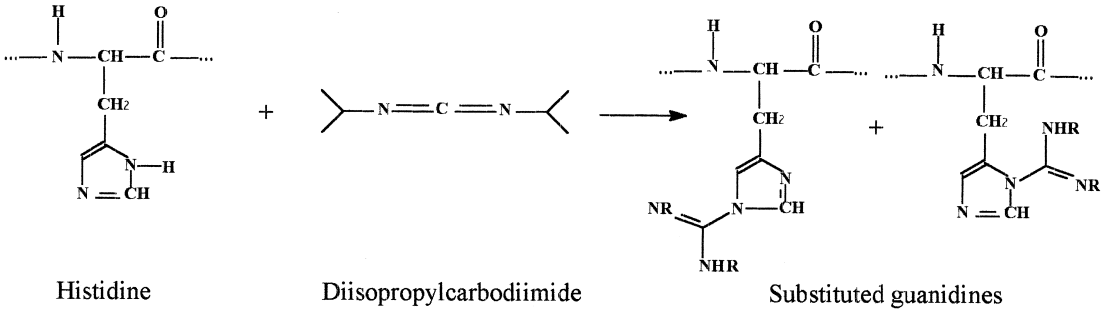


Fig. 10. Reaction of formation of a substituted guanidine.

4. Conclusions

The LC–ES-MS system has furnished fast and reliable information on the various products present in the crude of synthesis of leuprolide. The procedure followed for the synthesis of leuprolide appears to have resulted in partially deprotected peptide species, modified sequence peptide species due to the insertion of some amino acid, deletion peptide sequences, racemic peptides and modified species due to the reactivity of the side chains. So, the knowledge of the identities of the impurities present in the crude of synthesis allows one to optimize the synthesis procedure, improving the steps implicated, and fulfil the requirements for leuprolide commercialization as a therapeutic product.

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