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Liquid chromatography–electrospray mass spectrometry of multicomponent peptide mixtures
Characterization of a mixture from the synthesis of the hormone goserelin

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Abstract

In order to separate and characterize the target peptide and the side-product peptide compounds of a synthesis mixture of the peptide hormone goserelin, liquid chromatography coupled to high-flow electrospray ionization mass spectrometry (LC–ES-MS) has been used. Goserelin is an important drug with recognized therapeutical application for palliative treatment of prostatic and breast carcinomas. Stepwise solid-phase peptide synthesis commonly results in unwanted side-products associated with incomplete peptide chains. Consequently, this procedure requires extensive purification and characterization of the final synthesis mixture. The method of linear solvation energy relationships has been applied to optimize the proportion of organic modifier of the mobile phase used in the established LC method. On the other hand, ES-MS has allowed rapid and reliable identification of the target peptide and the other impurities present in the goserelin synthesis products.

Keywords: Linear solvation energy relationships; Pharmaceutical analysis; Peptides; Goserelin; Hormones

1. Introduction

Nowadays, the importance of peptides is evident in many fields. These biomolecules play an important role as hormones and neurotransmitters in the design of new drugs, in clinical diagnosis, etc., [1,2]. Discovery of new, biologically active peptides over the past few decades boosted the need for simplified and rapid methods for synthesizing them. Addressing these needs, in 1962 Merrifield introduced solid-phase peptide synthesis (SPPS) that, indeed, caused a revolution in the entire peptide field, and its influence spread to other areas. Goserelin is among several peptide hormones that were synthesized using this procedure.

Goserelin is a parenteral synthetic analog of luteinizing hormone-releasing hormone (LHRH) or gonadotropin-releasing hormone (GnRH). It is used for the palliative treatment of advanced prostatic carcinoma and for the management of endometriosis. Substitution of two amino acids normally found in GnRH leads to sustained activity that aids in hormonal control of prostate and breast carcinomas [3].

Peptide hormones are manufactured increasingly for various purposes. Those synthesized for therapeutic uses or preclinical investigations must be rigorously tested for their purity. Because peptides synthesized by the SSPS method may contain closely

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related peptide impurities that result from incomplete or side reactions, purification must be accomplished before the peptidic analog can be commercialized [4,5].

Liquid chromatography (LC) is the most versatile method for separation and purification of polypeptides [6–10]. Although a desired peptide separation may be obtained by trial and error, this may take many attempts with subsequent loss of time. The ability to predict the elution profiles of peptides on the basis of accurate quantitative relationships would greatly enhance the value of LC. In previous studies, the linear solvation energy relationship (LSER), based on the Kamlet–Taft multiparameter scale and on the E_T^N scale of polarity, was used to predict the retention of series of peptides [11], quinolones [12], diuretics [13] and anabolic steroids [14] in LC. This approach can be used to develop an LC methodology to separate target peptides from other, similar impurity peptide components, on the analytical scale and also on the preparative scale without lack of resolution.

The LSER formalism applied to chromatographic processes, and when a system with a fixed pair of solute and stationary phase is considered, can be expressed as follows [15–18]:

$$\log k = (\log k)_s + s\pi^*_m + a\alpha_m + b\beta_m \quad (1)$$

The independent term and the coefficients in Eq. (1) depend on solute and stationary phase parameters; the solvatochromic π^*_m parameter evaluates solvent dipolarity/polarizability [19]; and the solvatochromic parameters α_m and β_m evaluate solvent hydrogen bond acidity [20] and solvent hydrogen bond basicity [21] of the mobile phase, respectively. Taking into account that β_m values for acetonitrile (MeCN)–water mixtures, used here as mobile phases, are nearly constant [22,23] and that the observed correlation between the normalized Dimroth and Reichardt polarity parameter, E_T^N, [24] and π^* and α parameters, $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [25], Eq. (1) can be reduced to the single solvent parameter-dependent expression:

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

The linear correlation between the chromatographic retention, represented by the logarithm of the retention factor, and the E_T^N provides a useful tool for predicting retention due to the good linearity obtained [11–14] and because a suitable prediction of retention for a specific solute in a fixed stationary phase can be achieved from only two experimental measurements of k at two different mobile phase compositions. Because of its accuracy and simplicity, we judged it to be the best available as descriptor of retention as a function of percentage of organic solvent in the mobile phase.

Commercialization of therapeutic peptides not only requires purification but also characterization of the side-products present in the mixture of synthesis. Furthermore, this characterization allows one to improve the process of synthesis by suitable modification of those steps in which potentially by-products are produced.

LC coupled with mass spectrometry (MS) has proved to be a highly valuable technique for detailed structural characterization and purity evaluation of peptide mixtures and crude synthetic products [26–30]. Electrospray ionization (ES), a relatively gentle technique generally leads to the formation of multiply charged analyte ions, permits the analysis of very large, intact biomolecules and has become one of the most successful interfaces between LC and MS [31].

In this study, a mixture from the synthesis of goserelin has been examined. Firstly, the proportion of the organic modifier of the mobile phase was optimized by establishing relationships between Reichardt's E_T^N parameter of solvent polarity and the retention data. The synthesis mixture was then analyzed by LC–ES-MS in the chromatographic conditions optimized with this LSER methodology. The molecular masses of various side products within the mixture were determined, and, on the basis of these molecular masses, their sequence has been proposed.

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm, acetonitrile (Merck, Darmstadt, Germany) and dioxane (Merck) were of LC grade. Trifluoroacetic acid

(TFA), potassium bromide and potassium hydrogenphthalate (dried at 110°C before use) were all analytical grade obtained from Merck. The mixtures from the synthesis of goserelin [Pyr–His–Trp–Ser–Tyr–D-(^{t}Bu)Ser–Leu–Arg–Pro–AzGly–NH_2] used in this study were supplied by Lipotec (Barcelona, Spain). Goserelin mixtures were dissolved in the mobile phase at concentrations of 1 and 3 mg/ml, 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 through the solution. 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, 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 were measured with a Model 2002 potentiometer (±0.1 mV) (Crison Instruments, Barcelona, Spain) using an Orion 8102 Ross Combination pH electrode (Orion Research, Boston, MA, USA). The potentiometric system was calibrated with a standard reference solution of 0.05 mol/kg potassium hydrogenphthalate [18], whose reference pH values in the acetonitrile–water mixtures studied were previously assigned [32].

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 single 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 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. Dioxane (0.3 ml/min) was added post-column to improve sensitivity. The total flow was split to allow an effective flow of 60 μl/min into the source of the spectrometer. Instrument control and data analysis were performed using Masslynx application software from Micromass.

Mass calibration of the mass spectrometer was made using NaI. After calibration, a standard of goserelin was injected seven times to test the accuracy and precision of the mass measurements. The molecular mass obtained for goserelin was 1269.2, which is in good agreement with the expected 1269.4, and the relative standard deviation was 0.01%.

2.3. Procedures

2.3.1. LC–UV procedure

For the optimization of the mobile phase composition, different acetonitrile–water mixtures containing 0.1% (v/v) TFA were used. The acetonitrile percentage of these mixtures ranged from 24% to 30% (v/v). The flow-rate of mobile phase was 1 ml/min. Retention 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 by means of a potassium bromide solution 0.01% (w/v) in water. The calculated average t_0 in this composition range was 2.20 min. The retention times and the retention factors of the solutes were determined from three injections of 1 mg/ml solution of goserelin 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, and it ranged from 1.9 to 2.0.

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 a 0.1 mg/ml mixture of synthesis solution in MeCN–water (27:73), 0.1% (v/v) TFA, introduced directly into the ES source. Parameters were optimized in order to

obtain the best signal stability and the highest sensitivity of the target peptide, goserelin. Optimum conditions were as follows: flow-rate, 60 μl/min; capillary voltage 4000 V; counter electrode voltage, 250 V; sample cone voltage, 90 V; source temperature, 100°C; ion energy, 3 V. Fragmentation was negligible under this working conditions, which allowed correct identification of the molecular identities.

In order to improve the analytical signal obtained, post-column additions of several solvents at different flow-rates were made. The best results were obtained using dioxane at 0.3 ml/min.

2.3.2.2. Identification of side-products

For the identification of the side-products of the crude of synthesis, a 3 mg/ml solution of goserelin synthesis mixture was injected into the LC–ES-MS system, using MeCN–water (27:73, v/v) containing 0.1% (v/v) TFA as mobile phase (pH 1.94) with 0.3 ml/min of dioxane post-column addition. ES spectra of target peptide goserelin and associated side products were obtained in the positive ion mode 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

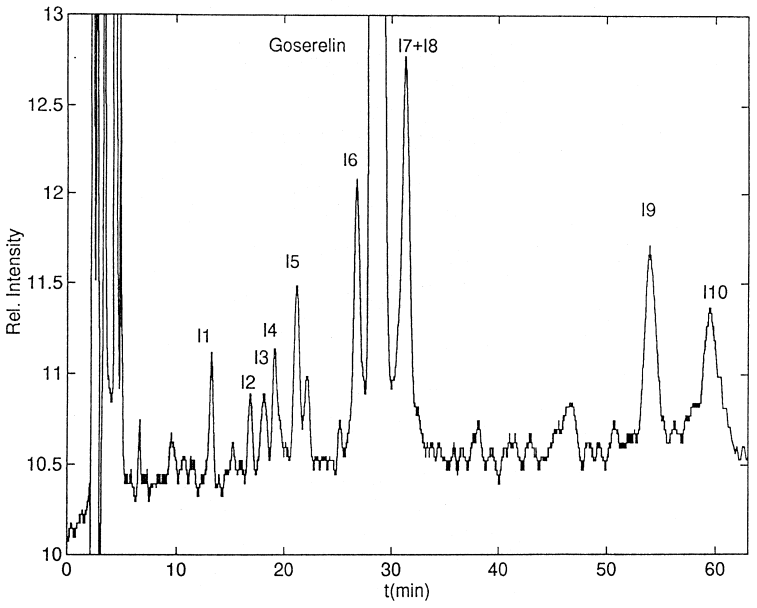
The synthetic product was first examined by analytical LC. The retention factor values, k, were obtained for the target peptide and for all the impurities at different percentages of acetonitrile from 24 to 30% (v/v) (Table 1). Owing to the large retention times for some of the substances studied, it has not been possible to obtain their retention factors in the whole acetonitrile–water range of mixtures. Selected UV chromatograms with mobile phases of MeCN–water (25:75) and (30:70) mixture, 0.1% TFA, are shown in Fig. 1. As it can be seen, chromatograms contain a major peak associated with the target peptide goserelin, as well as a number of minor peaks corresponding to unidentified peptide sequences, I1 to I13.

Chromatographic retention has been correlated with properties of the hydro–organic mixtures used as mobile phases; that is, the solvatochromic Reichardt's E_T^N parameter. Plots of log k of the substances studied here versus E_T^N values of acetonitrile–water systems are shown in Fig. 2. As can be observed and according to Eq. (2), log k and E_T^N correlate linearly ($r>0.99$) over the whole experimental range of acetonitrile content studied, which provides a good tool for predicting chromatographic retention of peptide derivatives. The use of Eq. (2) involves an important reduction of experimental retention data for the optimization of separation of solutes. Once the linearity of plots log k vs. E_T^N values has been verified, only two experimental measurements of retention factors for each compound considered at two different mobile phase compositions are sufficient to predict their retention behavior and hence for optimize their chromatographic separation and resolution in a fixed stationary phase.

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

MeCN (%, v/v)	E_T^N	Impurity I1	Impurity I2	Impurity I3	Impurity I4	Impurity I5	Impurity I6	Goserelin	Impurity I7	Impurity I8	Impurity I9	Impurity I10	Impurity I11	Impurity I12
24	0.881	0.8008	0.9160	0.9793	1.009	1.055	1.167	1.195	1.257	1.257	1.524	1.572	–	–
25	0.877	0.6943	0.8157	0.8520	0.8773	0.9298	1.041	1.068	1.115	1.115	1.365	1.409	–	–
26	0.873	0.5497	0.6449	0.6987	0.7286	0.7736	0.8784	0.9051	0.9324	0.9690	1.178	1.206	–	–
27	0.869	0.4339	0.5131	0.5808	0.6094	0.6523	0.7527	0.7791	0.7791	0.8448	1.031	1.052	1.309	1.387
28	0.865	0.3180	0.3826	0.4599	0.4853	0.5235	0.6534	0.6534	0.6534	0.7238	0.8955	0.8955	1.141	1.236
29	0.862	0.2068	0.2413	0.3385	0.3680	0.4035	0.5274	0.5274	0.5274	0.5925	0.7329	0.7329	0.9680	1.099
30	0.859	0.1348	0.1348	0.2667	0.2667	0.3040	0.4133	0.4133	0.4133	0.4780	0.5883	0.5883	0.7935	0.9527

a) Mobile phase: 25% MeCN : 75% H_2O, 0.1% TFA



b) Mobile phase: 30% MeCN : 70% H_2O, 0.1% TFA

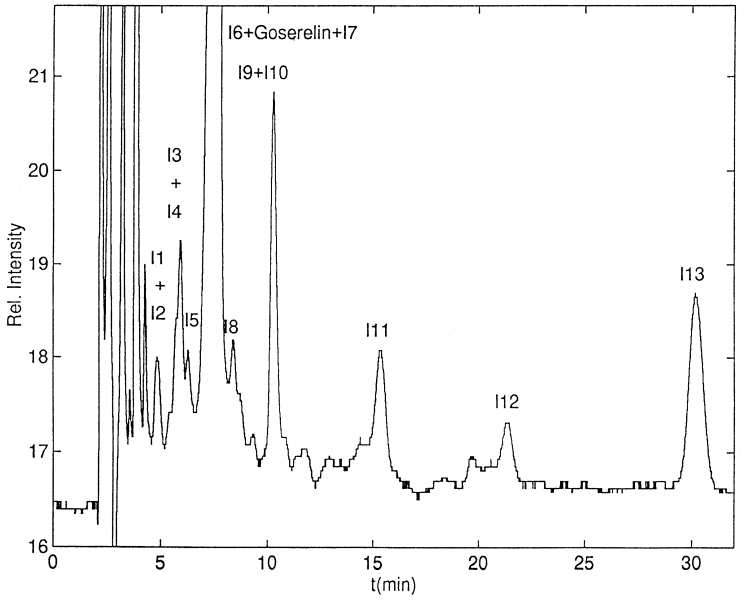


Fig. 1. UV chromatogram of a solution containing 1 mg/ml of a crude of synthesis of goserelin. (a) Mobile phase: MeCN–water (25:75), 0.1% (v/v) TFA. (b) Mobile phase: MeCN–water (30:70), 0.1% (v/v) TFA.

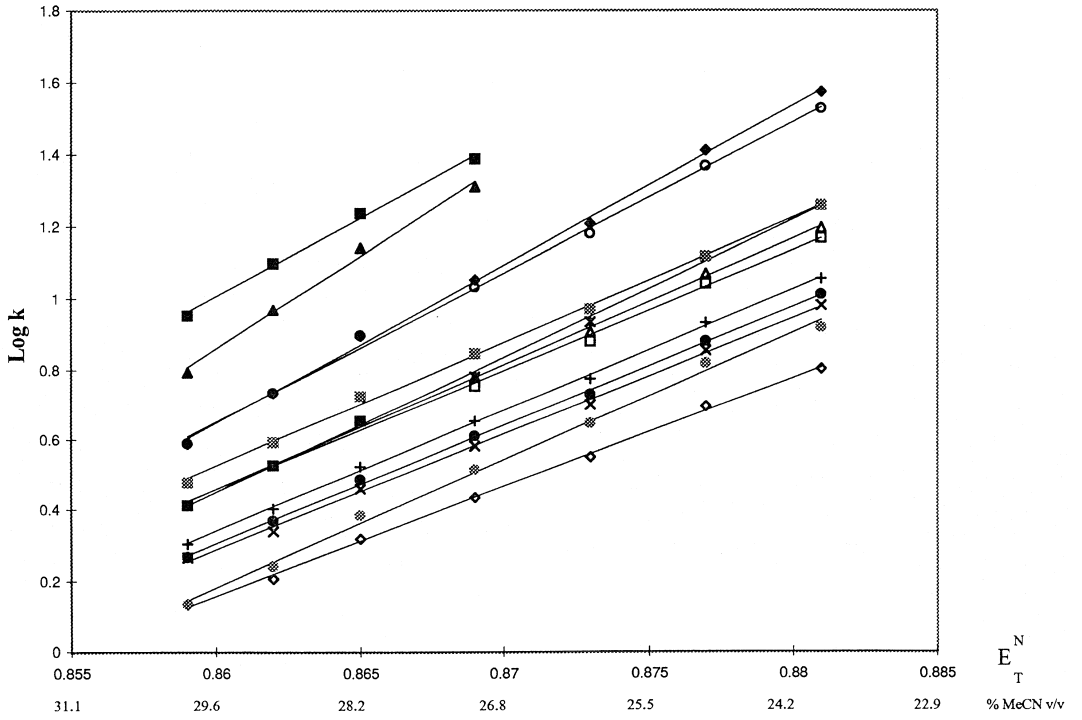


Fig. 2. Plots of log k of goserelin and impurities versus E_T^N parameters of the mobile phase: impurity I1 (◇), impurity I2 (▨), impurity I3 (×), impurity I4 (●), impurity I5 (+), impurity I6 (□), goserelin (△), impurity I7 (✱), impurity I8 (▨), impurity I9 (○), impurity I10 (♦), impurity I11 (▲) and impurity I12 (■).

In terms of fundamental chromatographic parameters, the resolution, R_s, between two adjacent peaks is given by:

$$R_s = \underbrace{\frac{1}{4}\cdot\sqrt{N_2}}_{\text{efficiency}} \cdot \underbrace{\left(\frac{\alpha-1}{\alpha}\right)}_{\text{selectivity}} \cdot \underbrace{\left(\frac{k_2}{1+k_2}\right)}_{\text{retention}} \quad (3)$$

Although the selectivity term is generally regarded as the most important in LC, to optimize resolution full attention must be given to all three terms in the fundamental resolution Eq. (3). Fig. 3 shows variations of R_s for solute pairs with the percentage of acetonitrile in the mobile phase. Only resolution between solutes whose separation was difficult was considered. Solid lines indicate resolution values obtained from two retention measurements using Eqs. (2) and (3), and points represent experimental resolution values obtained from the usual relation:

$$R_s = 2\cdot\frac{(t_{R2}-t_{R1})}{w_2+w_1} \quad (4)$$

where w_2 and w_1 are peak widths.

The concordance of the two sets of R_s values in Fig. 3 confirms accurate estimations of retention predictions and resolution via Eqs. (2) and (3) from only two experimental measurements per compound.

From Figs. 2 and 3, it can be concluded that good chromatographic separation between the target peptide and the impurities present in the crude can be achieved at percentages of acetonitrile in the mobile phase of 26–27% (v/v). On the other hand, the best resolution between goserelin and the adjacent impurities is achieved when the acetonitrile content in the mobile phase is 24%. Thus, the mobile phase recommended for the purification of Goserelin if preparative chromatography is used is acetonitrile–water (24:76), 0.1% TFA. For reliable identification of the target peptide and associate side-products by MS detection, an acetonitrile percentage of 27% (v/v) is to be preferred, owing to the improvement of the analytical response at higher acetonitrile percentages. This mobile phase composition provides a

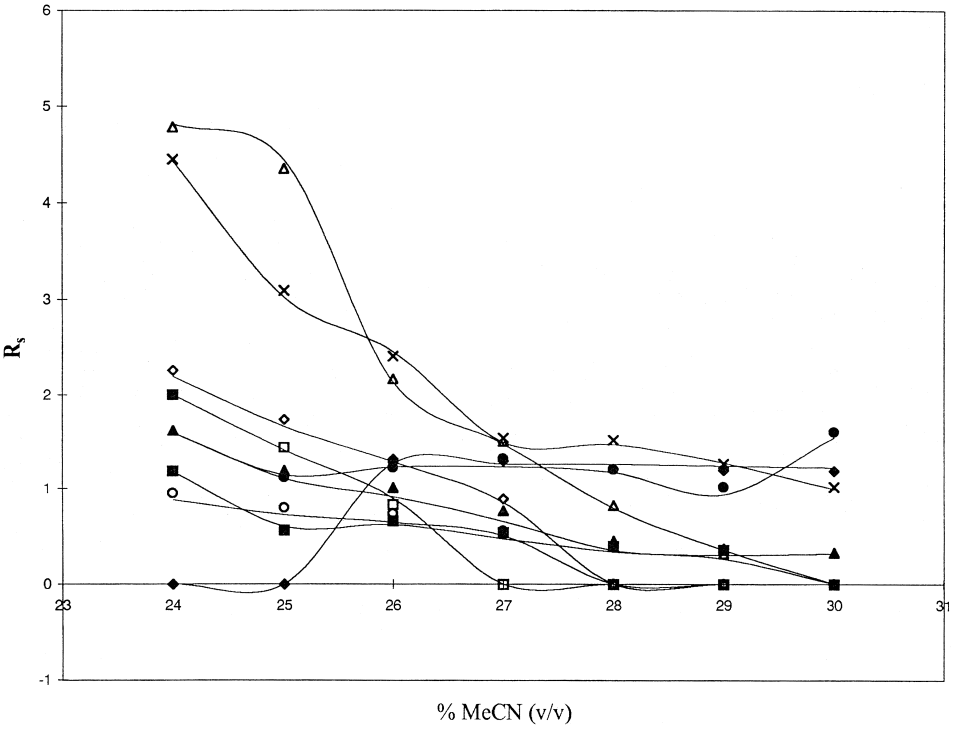


Fig. 3. Plots of resolution between adjacent pairs of solutes versus acetonitrile percentage in the mobile phase. Solid lines indicate theoretical values of resolution and points represent experimental values of resolution: impurity I1/impurity I2 (△), impurity I2/impurity I3 (●), impurity I3/impurity I4 (■), impurity I4/impurity I5 (▲), impurity I5/impurity I6 (×), impurity I6/goserelin (○), goserelin/impurity I7 (□), impurity I7/impurity I8 (♦) and impurity I9/impurity I10 (◇).

good separation among almost all the substances present in the synthesis mixture, allowing their characterization by MS without confusing overlaps.

3.2. LC–ES-MS

3.2.1. Characterization of the mixture of synthesis

LC–ES-MS was performed under the conditions described in the Experimental section. The effect of solvent composition on the detection of peptides in a complex mixture is critical. It has been observed that the use of high water percentages under ES conditions weakens the ES response because water does not allow an efficient droplet charging, and is less volatile and more difficult to spray than organic solvents [33,34]. The choice of an acetonitrile percentage of at least 27% (v/v) and the post-addition of a 20% (v/v) of dioxane provided a signal enhancement and allowed characterization of side-products. The total ion chromatogram of the crude of synthesis is given in Fig. 4. Besides the impurities assigned in Fig. 1 using UV detection, I1 to I13, other peaks detectable only by MS (A to F) appear in Fig. 4.

Figs. 5–7 show the spectra of the target peptide goserelin and also the spectra associated with some of the chromatographic peaks I1–I13 and A–F observed in the total ion current (TIC). The mass-to-charge ratios observed, the respective charged forms, and the average molecular masses calculated for each substance as well as the proposed sequences for each ion are summarized in Table 2. It can be observed in Table 2 that some chromatographic peaks contain several co-eluting impurities. The identification of these co-eluting impurities has been made from the data obtained at other acetonitrile percentages. In the same way, the impurity I13 has been identified from the ES data obtained at 29% (v/v) MeCN composition.

The method used to synthesize Goserelin provides

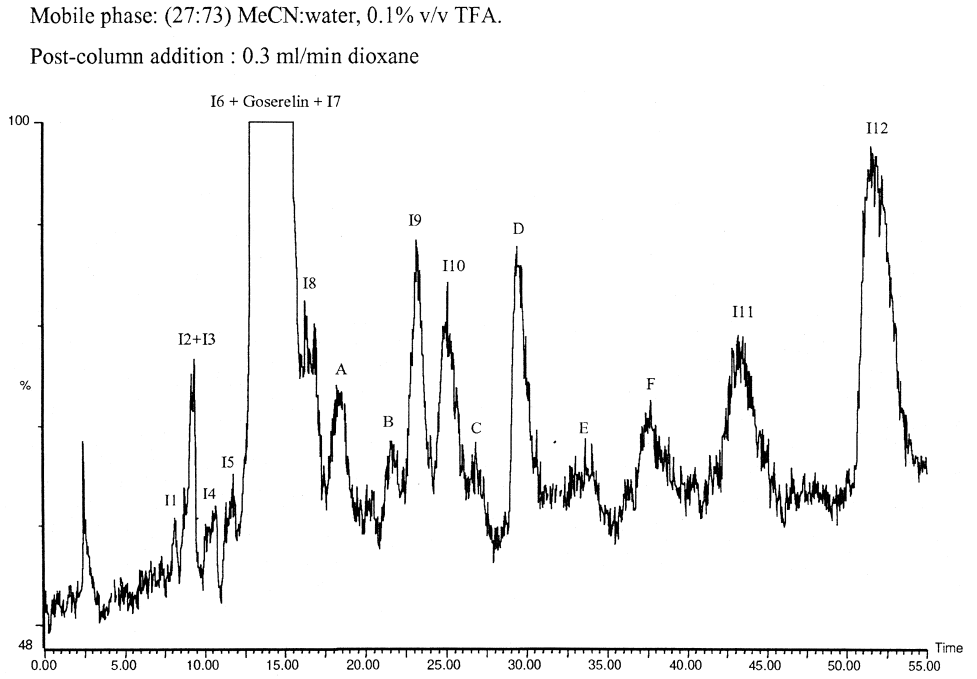


Fig. 4. Total ion current (TIC) chromatogram of the synthesis crude, with a mobile phase of MeCN–water (27:73), 0.1% (v/v) TFA, at optimal experimental conditions. Time scale in min.

useful information about the identity of the side products. Goserelin was prepared by a SPPS method, with the following protection scheme: 9-fluorenylmethoxycarbonyl (Fmoc) group to protect the α-amino group, 2-Cl-Trt (2-chlorotriphenylmethyl) group to protect the hydroxyl group of tyrosine side chain and Mmt (*p*-methoxytriphenylmethyl) group to protect the NH group in the imidazole ring of the histidine. After the last coupling, the peptide was cleaved from the resin and deprotected by acidolytic treatment, and finally amidated with the group azylglycinamida to obtain the final product.

3.2.2. Target peptide

The electrospray spectrum of the target peptide, eluting at $t_R = 13.27$ min, is shown in Fig. 5. The ion at m/z 1270.1, corresponding to $[M+H]^+$, and that at m/z 635.5 corresponding to $[M+2H]^{2+}$, associated with a molecular mass of 1269.1 are in good agreement with the calculated molecular mass of the goserelin, 1269.4.

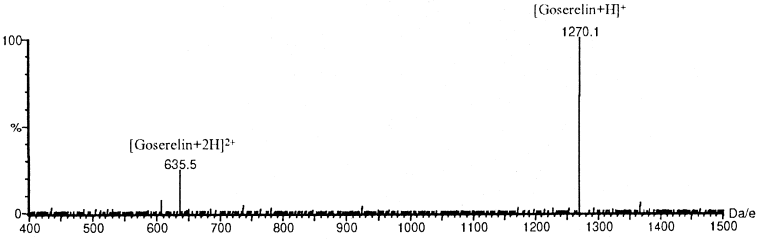
3.2.3. Impurities

The present identification of the side products [35,36] is solely based on the observed mass differences between the mass of goserelin, 1269.4, and the mass of the side product [37].

3.2.3.1. Racemization products

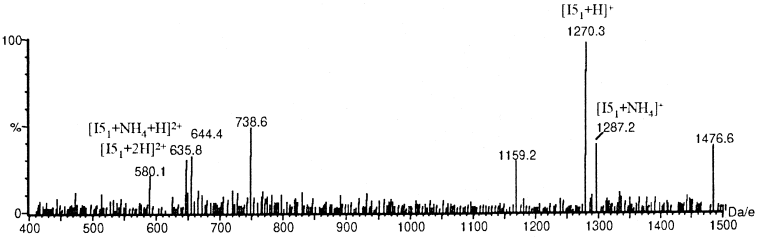
Racemization or isomerization of amino acids during the SPPS has been observed frequently [38], mainly in the activation and coupling steps. The use of a high concentration of reactives to secure high coupling rates and of some additives normally allows minimization of this undesired reaction. A racemate of goserelin has been identified in the chromatographic peak I5, the spectrum of which contains ions $m/z = 1270.3$ and $m/z = 635.8$, associated with a molecular mass of 1269.5 ($\Delta M = -0.1$).The $[M+NH_4]^+$ and $[M+NH_4+H]^{2+}$ adduct ions are also present in the spectrum. The remainder of the m/z ratios present in the spectrum will be assigned to other impurities afterwards.

a) TARGET PEPTIDE Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-AzGly-NH_2



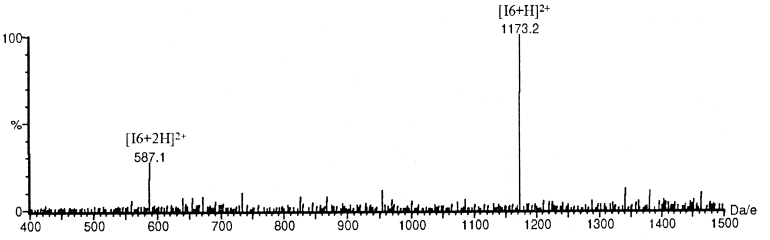
b) RACEMATE

• **I5**

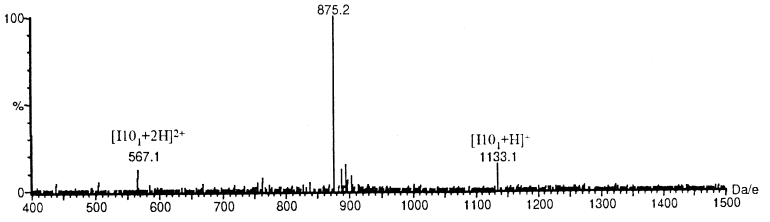


c) DELETION SEQUENCES

• **I6** Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-[ ]-AzGly-NH_2



•**I10: I10$_1$** Pyr-[ ]-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-AzGly-NH_2



•**I5$_3$** [ ]-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-AzGly-NH_2

•**I8$_1$** Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-[ ]-NH_2

•**E$_2$** Pyr-His-Trp-Ser-Tyr-D-Ser(^{t}Bu)-Leu-Arg-Pro-NH-NH_2

Fig. 5. Electrospray mass spectra associated with the chromatographic peaks indicated and structures associated with: (a) goserelin, (b) racemate of goserelin and (c) deletion sequences. Da/e = m/z.

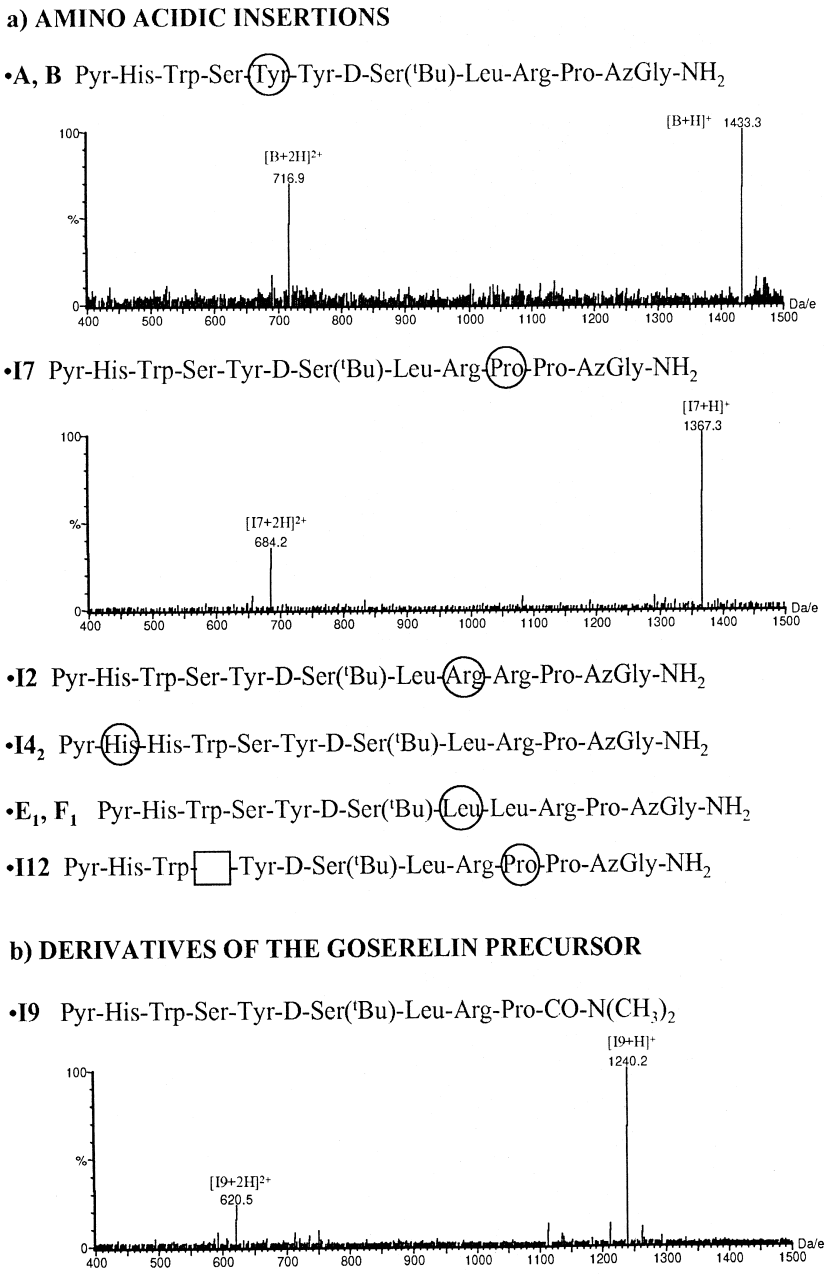


Fig. 6. Electrospray mass spectra associated with the chromatographic peaks indicated and structures associated with: (a) products of amino acidic insertions and (b) derivative of the goserelin precursor. Da/e=m/z.

3.2.3.2. Deletion sequences

Incomplete removal of blocking groups lead to the formation of chains from which one of the amino acid residues is absent. Such materials have been designated ''failure sequences'' or ''deletion sequences'' [39]. Impurities $I5_3$, I6, $I8_1$ and $I10_2$ (chromatographic peaks I5, I8 and I10 show the co-elution of several impurities) have been attributed

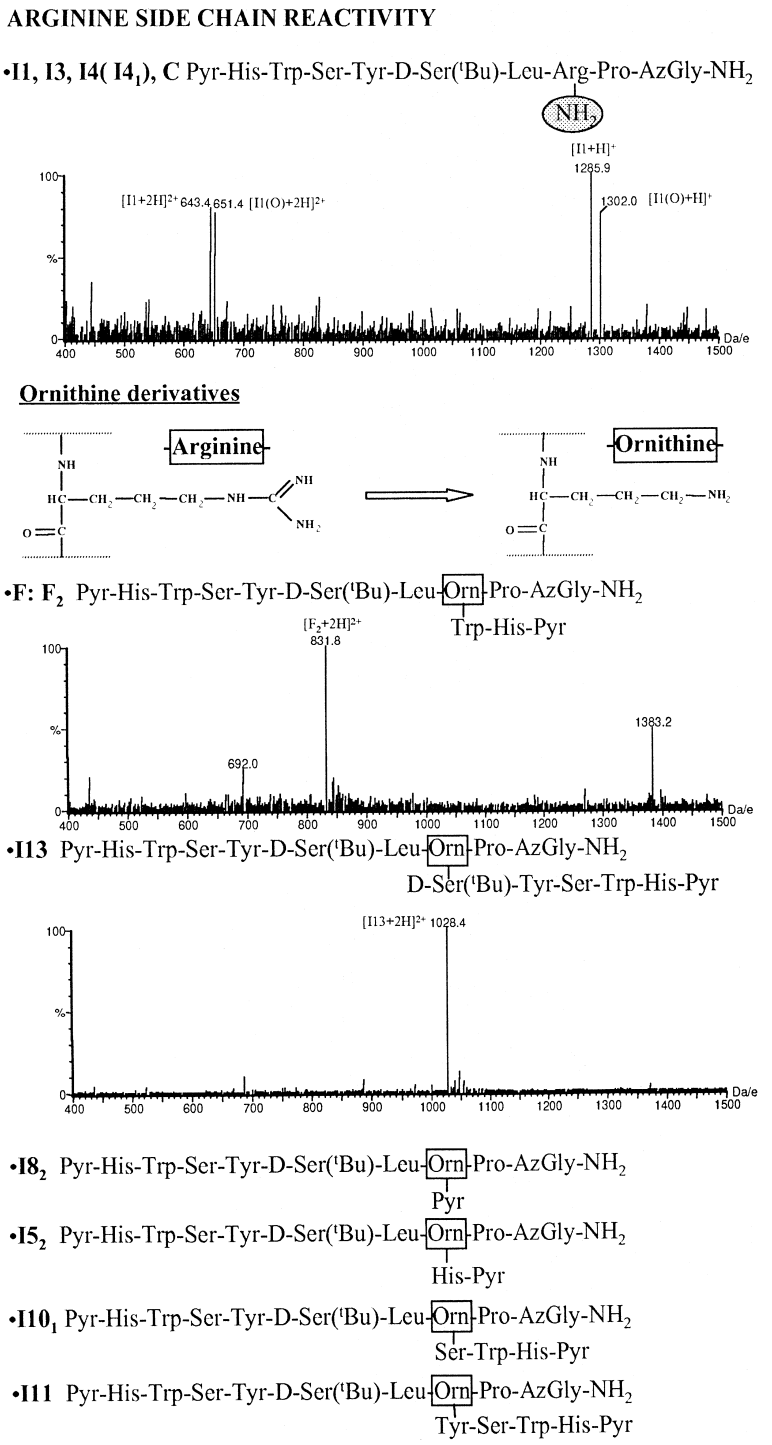


Fig. 7. Electrospray mass spectra associated with the chromatographic peaks indicated and structures associated with the products of arginine side chain reactivity. Da/e = m/z.

Table 2
Measured relative molecular masses (M_r) and proposed sequences associated with the TIC peaks in Fig. 4

Chromatographic peak	Impurity	t_R (min) 27% MeCN	Observed m/z, $[M+2H]^{2+}$ $[M+H]^{+}$	Measured M_r	ΔM	Proposed sequence
I1	I1	8.10	643.4 1285.9 $[M(O)+2H]^{2+}$ $[M(O)+H]^{+}$ 651.4 1302.0	1284.9	+15.5	Addition of a NH_2 group to the side chain of arginine residue
I2+I3	I2	9.34	713.7 1426.2	1425.3	+155.9	Goserelin+arginine
	I3	9.34	643.2 1285.7	1284.5	+15.1	Addition of a NH_2 group to the side chain of arginine residue
I4	$I4_1$	10.40	643.2 1285.7	1284.5	+15.1	Addition of a NH_2 group to the side chain of arginine residue
	$I4_2$		704.3 1407.2	1406.4	+137.0	Goserelin+histidine
I5	$I5_1$	11.75	635.8 1270.3 $[M+NH_4+H]^{2+}$ $[M+NH_4]^{+}$ 644.4 1287.2	1269.5	−0.1	Goserelin racemate
	$I5_2$		738.6 1476.6	1475.4	+206.0	Ornithine derivative+(His–Pyr)
	$I5_3$		580.1 1159.2	1158.2	−111.2	Goserelin–pyroglutamic acid
I6 +	I6	13.27	587.1 1173.2	1172.2	−97.2	Goserelin–proline
Goserelin +	Goserelin	13.27	635.5 1270.1	1269.1	−0.3	
I7	I7	13.27	684.2 1367.3	1366.4	+97.0	Goserelin+proline
I8	$I8_1$	16.50	606.4 1212.2	1211.0	−58.4	Goserelin–(AzGly)
	$I8_2$		669.9 1339.2	1338.0	+68.6	Ornithine derivative+(Pyr)
A	A	18.48	717.1 1433.3	1432.3	+162.9	Goserelin+tyrosine
B	B	21.52	716.9 1433.3	1432.0	+162.6	Goserelin+tyrosine

Table 2. Continued

Chromatographic peak	Impurity	t_R (min) 27% MeCN	Observed m/z, $[M+2H]^{2+}$ $[M+H]^{+}$	Measured M_r	ΔM	Proposed sequence
I9	I9	23.12	620.5 1240.2	1239.1	−30.3	Amidation of the acid precursor
I10	$I10_1$	25.04	875.2 –	1748.4	+479.0	Ornithine derivative+(Ser–Trp–His–Pyr)
	$I10_2$		567.1 1133.1	1132.2	−137.2	Goserelin–histidine
C	C	26.80	643.1 1285.2	1284.2	+14.8	Addition of a NH_2 group to the side chain of arginine residue
E	E_1	34.04	692.0 1383.5	1382.3	+112.9	Goserelin+leucine
	E_2		614.1 1226.9	1226.0	−43.4	Formation of an acylhydrazyn
F	F_1	37.72	692.0 1383.2	1382.1	+112.7	Goserelin+leucine
	F_2		831.8 –	1661.6	+392.2	Ornithine derivative+(Trp–His–Pyr)
I11	I11	43.12	956.8 –	1911.6	+642.2	Ornithine derivative+(Tyr–Ser–Trp–His–Pyr)
I12	I12	51.44	640.4 1280.2	1279.0	+9.6	Goserelin+proline–serine
I13	I13	50.7 (29% MeCN)	1028.4 –	2054.8	+785.4	Ornithine derivative+[D-Ser(^{t}Bu)–Tyr–Ser–Trp–His–Pyr]

Not identified: D

to deletion sequences. As an example, spectra of I6 and I10 are shown in Fig. 5. The spectrum associated with impurity I6 exhibits m/z of 1173.2 $[M+H]^{+}$ and 587.1 $[M+2H]^{2+}$, yielding the molecular mass of 1772.2. The difference between its molecular mass and the mass of goserelin is −97.2, corresponding to the proline residue. This difference indicates the presence of a modified goserelin chain due to the absence of a proline.

In the same way, chromatographic peak I10 contains the ions of m/z 1133.1 $[M+H]^{+}$ and 567.1 $[M+2H]^{2+}$ in its spectrum, corresponding to a molecular mass of 1132.2 ($\Delta M = -137.2$), which indicates the absence of a histidine residue. Chromatographic peak I5 contains a deletion sequence that implies the loss of the Pyr residue, and impurity $I8_1$ is attributed to a deletion sequence with the loss of the –AzGly group (spectra not shown). On the other hand, the loss of the C-terminal semicarbazide group ($\Delta M = -43$) leads to the formation of the acylhidrazine, impurity E_2. This side-product has also been observed by other authors as a degradation product of the goserelin analog [36].

3.2.3.3. Amino acidic insertions

The use of an excess of equivalents in the coupling step to ensure the maximum coupling efficiency leads to the occasional insertion of an

additional amino acid in the sequence. This is the case of impurities I2 (additional arginine), A and B (additional tyrosine, isomers) and I7 (additional proline). The chromatographic peak I4 also contains a side-product which corresponds to the insertion of an additional histidine ($I4_2$) and chromatographic peaks E and F contain impurities attributed to the additional presence of a leucine (E_1 and F_1). Fig. 6 shows the spectra of some selected side-products associated with peaks A, B and I7 and the structures of all the impurities associated with a insertion sequence. Impurity I12 has been tentatively attributed to a sequence that contains an additional proline but lacks a serine residue.

3.2.3.4. Acid precursor

Before the synthesis of goserelin is completed by means of an amidation, the acid precursor was cleaved from the resin. This substance has not been found as impurity in our mixture but a derivative of the acid precursor has appeared in the chromatographic peak I9. Its spectrum, shown in Fig. 6, contains ions m/z 1240.2 $[M+H]^+$ and 620.5 $[M+2H]^{2+}$, corresponding to a molecular mass of 1239.1. The difference between this mass and the molecular mass of the acid precursor ($M=1212.4$) is of 26.6, attributed to the formation of the dimethylamide from the acid precursor. The $-N(Me)_2$ group is supplied by the dimethylformamide or the DIEA (*N*,*N*-diisopropylethylamine) used in the synthesis procedure.

3.2.3.5. Arginine side chain reactivity

Individual amino acid residues can undergo undesired reactions in their side chains such as alkylations, oxidations, cyclizations, and substitutions [36,40]. Chromatographic peaks I1, I3 and C show similar mass spectra, Fig. 7, with m/z ratios $\approx$1285.5 $[M+H]^+$ and 643 $[M+2H]^{2+}$ yielding a molecular mass of approximately 1284.5, that differs from goserelin molecular mass by about 15. Impurity $I4_1$ also shows this charge-state distribution. This difference has been attributed to the addition of a NH_2 group to the side chain of arginine residue. The singly and doubly charged ions 1302.0 and 651.4, respectively, correspond to the oxidation of this impurity in the electrospray source.

3.2.3.6. Ornithine derivatives

Among the side reactions that arginine can undergo, there is a particular reaction that gets great importance in our case. Fragmentation of the side chain of arginine takes place, and it is transformed into the ornithine residue (Fig. 7), with an amino group unprotected that allows the growth of the peptide sequence in two places. In each coupling step the amino acid can be linked to the main sequence and to the side chain of ornithine. Up to six impurities ($I8_2$, $I5_2$, F_2, $I10_1$, I11 and I13) can be attributed to this side reaction. Some selected spectra together with the structure of these derivatives are shown in Fig. 7.

4. Conclusions

The separation between the target peptide goserelin and undesired side products in the crude of synthesis has been optimized applying the LSER method. The assessment of the analytical scale conditions allows preparative scale purification of the target peptide. On the other hand, LC–ES-MS provides an efficient analytical tool for reliable characterization of the target peptide and associated impurities. The knowledge of the side-products identity allows the chemist to improve the synthetic procedures by suitable modification of conflictive steps and to fulfil the necessary requirements for goserelin commercialization as a peptide of therapeutic interest.

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