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Investigation of synthetic peptide hormones by liquid chromatography coupled to pneumatically assisted electrospray ionization mass spectrometry: analysis of a synthesis crude of peptide triptorelin

V. Sanz-Nebot*, I. Toro, A. Castillo and J. Barbosa

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

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Triptorelin, a synthetic peptide hormone used in the treatment of prostate cancer by means of reduction in the action of male hormone testosterone, is studied here. The synthetic procedure commonly results in unwanted side products that require extensive purification and characterization of the synthesis mixture. The chromatographic separation of triptorelin from the crude mixture was developed by applying the linear solvation energy relationship (LSER) methodology previously developed, to optimize the composition of the mobile phase in order to avoid lengthy empirical optimization procedures. Electrospray ionization mass spectrometry coupled to liquid chromatography (LC/ES-MS) was used to obtain reliable information on the inevitable side products. The knowledge of the identity of these impurities allows fast optimization of the synthetic procedure and also the therapeutic use of triptorelin peptide hormone. Copyright © 2001 John Wiley & Sons, Ltd.

Triptorelin (5-oxoPro-Pro-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2) is a synthetic peptide used in hormonal therapy in the treatment of prostate cancer. It has the effect of inhibiting the production of a hormone from the pituitary gland leading to a reduction in the male hormone testosterone. Prostate cancer is often sensitive to the levels of these hormones in the body, and reducing their levels may result in a slowing of the growth of the cancer.[1,2]

Peptides of pharmaceutical interest in the molecular mass range of 500–1500 Da, like triptorelin, are nowadays synthesized using solid phase peptide synthesis (SPPS). However, the final product not only contains the target peptide sequence, but also contaminating species that differ in subtle ways such as minor deletions, incomplete deprotected side chains, fragmentation at unexpected residues, and residue adducts as a consequence of improper resin cleavage.[3] Careful analysis of the full synthetic products, and elucidation of degradation products and by-products, are most important for guaranteeing the appropriate quality of the commercial product.[4,5]

Liquid chromatography (LC) has proved very versatile in aiding the isolation of peptides from complex synthetic mixtures.[6–10] In order to avoid lengthy empirical procedures to optimize separation of unwanted products associated with the peptide triptorelin from a synthesis crude, the ability to predict the elution profiles of peptide mixtures is of great advantage. In previous studies, linear solvation energy relationships (LSER), based on the multiparameter scale and on the E_T^N scale of polarity, have been used to predict the retention of series of peptides,[11,12] quinolones,[13] diuretics[14] and anabolic steroids.[15]

The LSER formalism applied to chromatographic processes, for a system with fixed stationary phase, can be expressed as follows:[16–19]

$$\log k = (\log k)_0 + s\pi^*_m + a\alpha_m + b\beta_m \quad (1)$$

The independent term k_0 and the coefficients s, a, b in Eqn. (1) for the retention factor k depend on the solute and stationary phase parameters; the solvatochromic π^*_m parameter evaluates solvent polarity/polarizability,[20] and the solvatochromic parameters α_m and β_m evaluate solvent hydrogen bond acidity[21] and basicity[22] of the mobile phase, respectively. Taking into account that β_m values for acetonitrile/water mixtures (used here as mobile phases) are nearly constant,[23,24] and also the observed correlation[26] between the normalized Dimroth and Reichardt polarity parameter[25] E_T^N and the π^*_m and α_m parameters:

$$E_T^N = 0.009 + 0.415\pi^*_{m^*} + 0.465\alpha_m$$

then Eqn. (1) can be reduced to the single solvent parameter-dependent expression:

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

The correlation (Eqn. (2)) between the chromatographic retention, represented by the logarithm of the retention factor and the parameter E_T^N provides a useful tool for predicting retention due to the good linearity thus obtained[11–15] and because a suitable prediction of retention for a specific solute in a fixed stationary phase can be achieved

**Correspondence to*: V. Sanz-Nebot, Departament de Química Analítica Universitat de Barcelona, Diagonal 647, 08028, Barcelona, Spain.
E-mail: nebot@zeus.qui.ub.es

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from only two experimental measurements of k at two different mobile phase compositions. Because of its accuracy and simplicity, we judged Eqn. (2) to be the best available descriptor of retention as a function of percentage of organic solvent in the mobile phase.

Commercialization of therapeutic peptides requires not only purification, but also characterization of the side products present in the synthesis crude in order to eliminate or minimize their production in the future. This characterization allows improvements in the synthetic procedure by suitable modification of those steps in which by-products are potentially produced. Liquid chromatography coupled to mass spectrometry using an electrospray ionization interface (LC/ES-MS) has proved to be highly efficient for fast and reliable analysis and characterization of crude synthetic products.[27–32]

Here, a synthesis crude of triptorelin [CAS name: luteinizing hormone-releasing factor, 6-D-tryptophan] has been analyzed by LC/ES-MS. First, the proportion of organic modifier of the mobile phase was optimized by establishing relationships between Reichardt's E_T^Nsolvent polarity parameter and the retention data. Then, the synthesis crude of triptorelin was analyzed by LC/ES-MS using the chromatographic conditions optimized by the LSER method. The molecular masses of several by-products from the synthesis crude have been determined, and the impurities have been identified on the basis of these molecular masses.

EXPERIMENTAL

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 hydrogen phthalate were all analytical grade obtained from Merck. The synthesis crudes were stored in a freezer at 0 °C when not in use. Working solutions of the synthesis crude were prepared at concentrations of 1 and 3 mg/mL for LC-UV and LC/ES-MS analysis, respectively, using the mobile phase as solvent. 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).

Apparatus

LC-UV

The chromatographic equipment consisted of an ISCO Model 2350 (Lincoln, NE, USA) pump, an injection valve with a 10-μL sample loop, and a variable wavelength V^4 absorbance detector (ISCO). The chromatographic system was controlled by ChemResearch Chromatographic Data Management System Controller Software (ISCO) on a personal computer. A Kromasil C_8 (5 μm) column 250 × 4.6 mm i.d. (BC Aplicaciones Analíticas, S.A. Barcelona, Spain) was used at room temperature.

The emf values used to evaluate the pH of the mobile phase were measured with a potentiometer (±0.1 mV) Model 2002 (Crison Instruments S.A., Barcelona, Spain) using an Orion 8102 ROSS combination pH electrode (Orion Research Incorporated, Boston, MA, USA). The potentiometric system was calibrated using a standard reference solution of potassium hydrogen phthalate (0.05 mol/kg) whose reference pH values in the acetonitrile/water mixtures studied had been assigned previously.[33]

LC/ES-MS

LC/ES-MS measurements 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 (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 (300–400 L/h) gas.

Separation was performed on a Kromasil column (250 × 4.6 mm i.d.) at room temperature and with 1 mL/min flow rate. The total flow was split to allow an effective flow of 50 μL/min into the source of the spectrometer. Instrument control and data analysis were performed using MassLynx application software from Micromass (Manchester, UK). Generally, the mass spectral data are the average of three separate measurements.

The calibration of the mass spectrometer was carried out using NaI. After calibration a standard of triptorelin was injected five times to test the accuracy and precision of the mass measurement. The (isotope-averaged) molecular mass obtained for triptorelin was 1311.2 Da, which is in good agreement with the expected value of 1311.5 Da, and the relative standard deviation was 0.02%.

Procedures

LC-UV procedure

The solutions used as mobile phase in order to optimize the mobile phase composition were composed of acetonitrile/water mixtures containing 0.1%(v/v) trifluoroacetic acid, with pH of the mobile phase 1.9–2.0, at several concentrations of acetonitrile from 23% to 29% (v/v). The Kromasil C_8 column was equilibrated with new mobile phase conditions for 30 min. All chromatograms were measured at room temperature.

The hold-up time, t_0, was established for every mobile phase composition using potassium bromide solution (0.01% (w/v) in water) and monitoring the signal at 200 nm. The retention times and the retention factors of the solutes were determined from three injections of 1 mg/mL solution of triptorelin crude at each mobile phase composition considered, monitoring the signal at 220 nm. The pH was measured in each mixed mobile phase used for the chromatographic separation.

LC/MS procedure

Optimization of the ES parameters. The source and analyzer parameters were optimized using electrospray ionization of a 50-ppm crude solution in (29:71) MeCN/water, 0.1% TFA, introduced directly into the ES source, working in full scan mode (*m/z* 500–1500).

Parameters were optimized in order to obtain the best signal stability and the highest sensitivity of the target

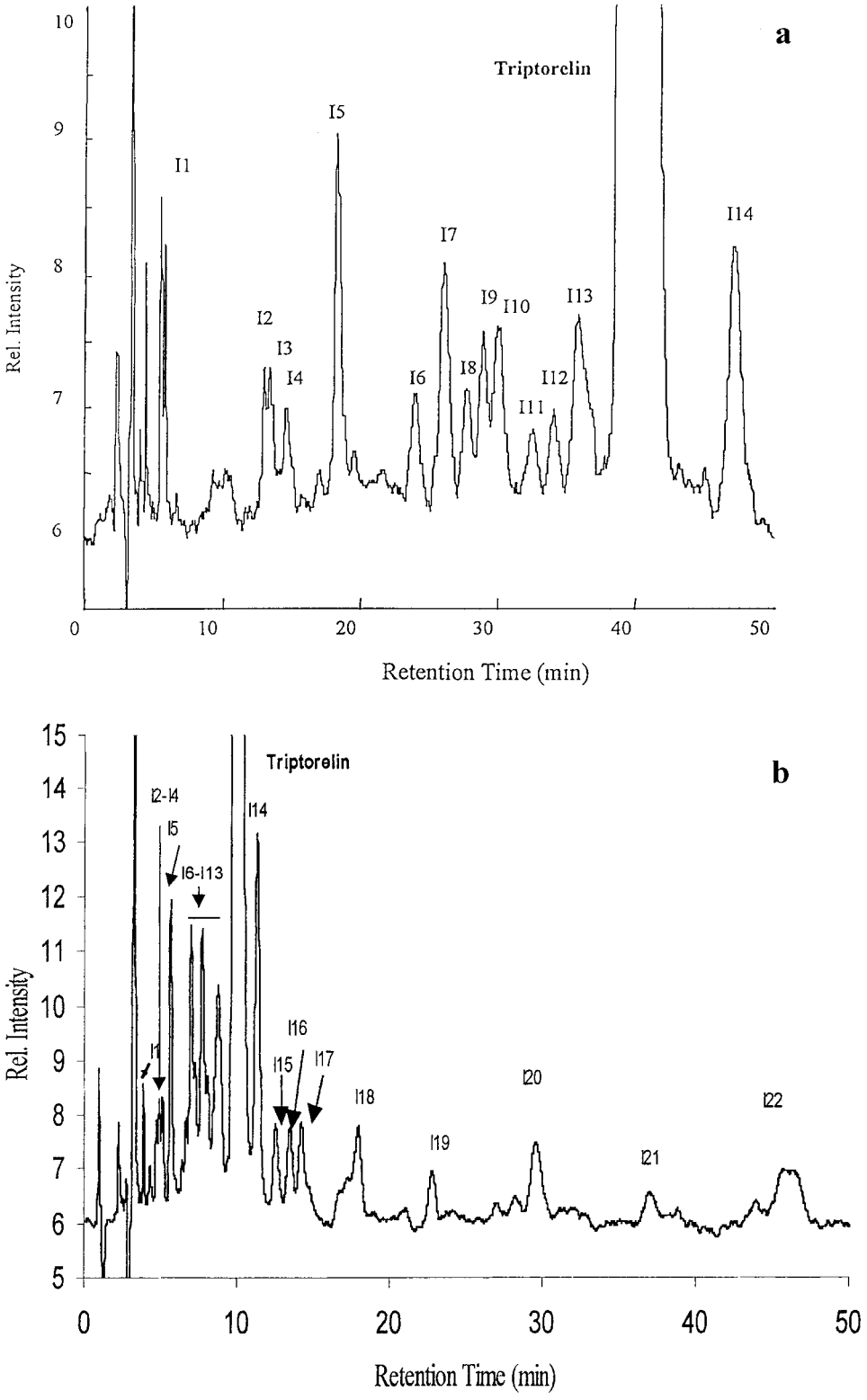

Figure 1. UV chromatograms of a solution containing 1mg/mL of synthesis crude of peptide triptorelin, with a mobile phase of (a) (24:76)% (v/v) acetonitrile/water, 0.1% TFA and (b) (29:71)% (v/v) acetonitrile/water, 0.1% TFA.

peptide triptorelin. The optimum conditions were: drying nitrogen gas flow (400 L/h); ES nebulizing nitrogen gas flow (20 L/h); electrospray probe (capillary) voltage (4.25 kV); counter electrode (HV lens) voltage (0.5 kV); sample cone voltage (110 V); source temperature (90 °C), low mass resolution (10.5), high mass resolution (11.0) and scan cycle time (2.5 s).

Identification of side products. For identification of side products in the synthesis crude, a 3 mg/mL solution of

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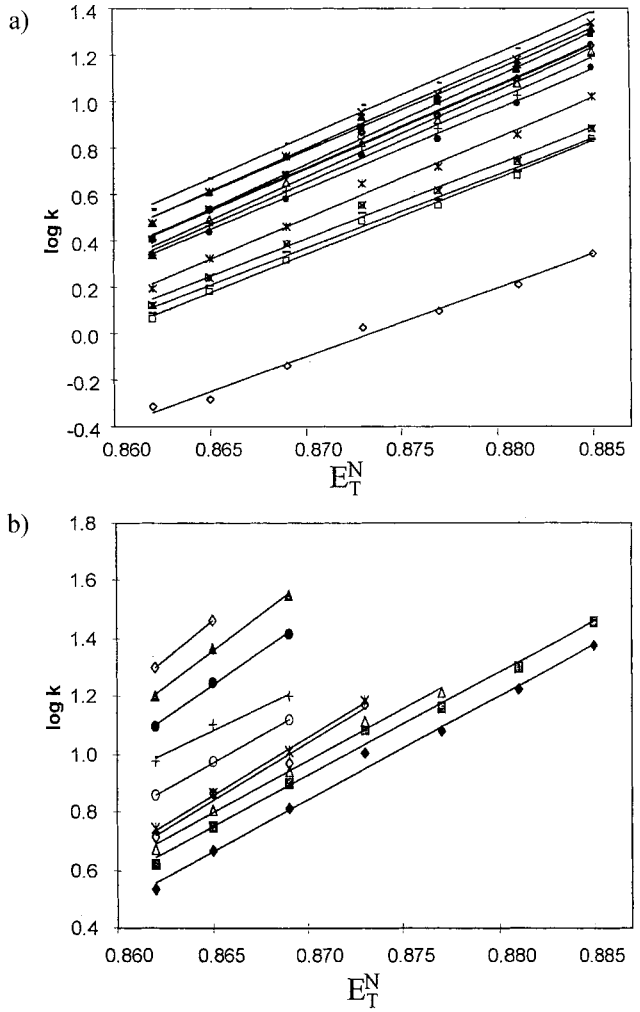
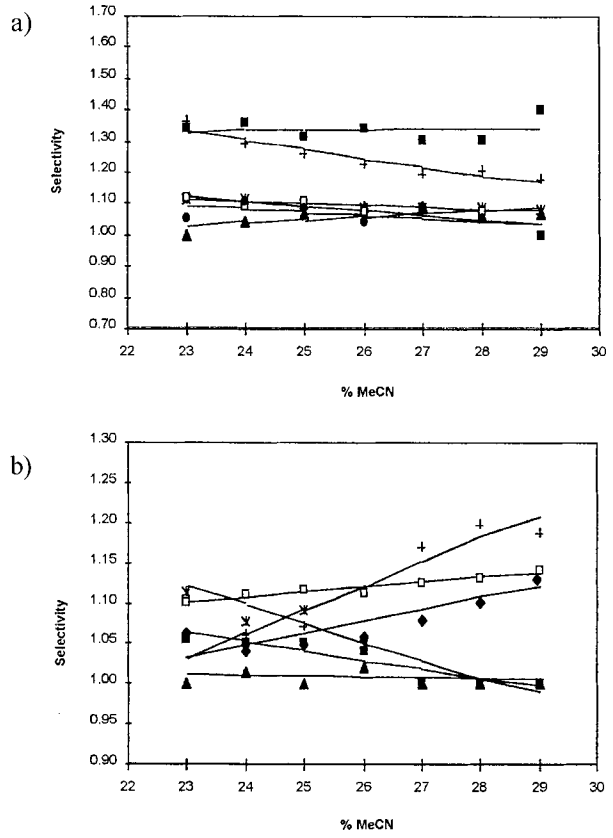


(a) I1 (◊), I2 (□), I3 (—), I4 (⊠), I5 (*), I6 (●), I7 (+), I8 (Δ), I9 (O), I10 (◆), I11(■), I12 (▲), I13 (X), Triptorelin (-).
(b) Triptorelin (◆), I14 (■), I15(Δ), I16 (◆), I17 (*), I18 (O), I19 (+), I20 (●), I21 (▲), I22 (◊).

Figure 2. Plots of log k for triptorelin and impurities vs. E_T^N parameters of the mobile phase.



(a) I2/I1 (◆), I3/I2 (▲), I4/I3 (*), I5/I4 (+), I6/I5 (■), I7/I6 (□), I8/I7 (O),
(b) I9/I8 (◆), I10/I9 (▲), I11/I10 (*), I12/I11 (+), I13/I12 (■), Tri/I13 (□).

Figure 3. Variation in selectivity values for compound pairs with acetonitrile percentage. Solid lines indicate predicted values of selectivity from Eqn. (2) and points represent experimental values of selectivity.

triptorelin crude was injected into the LC/ES-MS system using MeCN/water (29:71) containing 0.1% TFA (v/v) as mobile phase (pH 1.9–2.0). ES spectra of the target peptide triptorelin and of the associated side products were obtained in positive ion mode using the optimum conditions of the mass spectrometer described in the Experimental section.

RESULTS AND DISCUSSION

The synthetic product was first examined by analytical LC with UV detection. The retention factor values (k) were obtained for the target peptide and for all the impurities at different percentages of MeCN from 23% to 29% (v/v).

Owing to the large retention times for some of the substances studied it was not possible to obtain their retention factors over the whole range of acetonitrile/water mixtures. Selected UV chromatograms with mobile phases of (24:76) and (29:71) (v/v) acetonitrile/water mixtures, 0.1% TFA, are shown in Fig. 1. The resulting UV chromatogram contains a major peak corresponding to the target peptide (triptorelin), as well as a number of peaks corresponding to unidentified peptides, impurities I1 to I22.

To optimize the composition of the mobile phase, Reichardt's E_T^N polarity parameter (known[25] for the whole range of composition of MeCN/water) was related to the retention factors of the target peptide and associated side products to be separated using the LSER methodology (Eqn. (2)). Plots of log k for the substances studied here versus E_T^N values of MeCN/water systems are shown in Fig. 2. In accord with Eqn. (2), log k and E_T^N correlate linearly ($r > 0.99$) over the whole experimental range of acetonitrile content studied, thus providing a good tool for predicting chromatographic retention of peptide compounds. Equation (2) provides an important basis for reduction of experimental retention data for optimizing separation of solutes. Once the linearity of plots of 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 enough to predict their retention behaviour and hence to optimize their chromatographic separation and resolution using a fixed stationary phase.

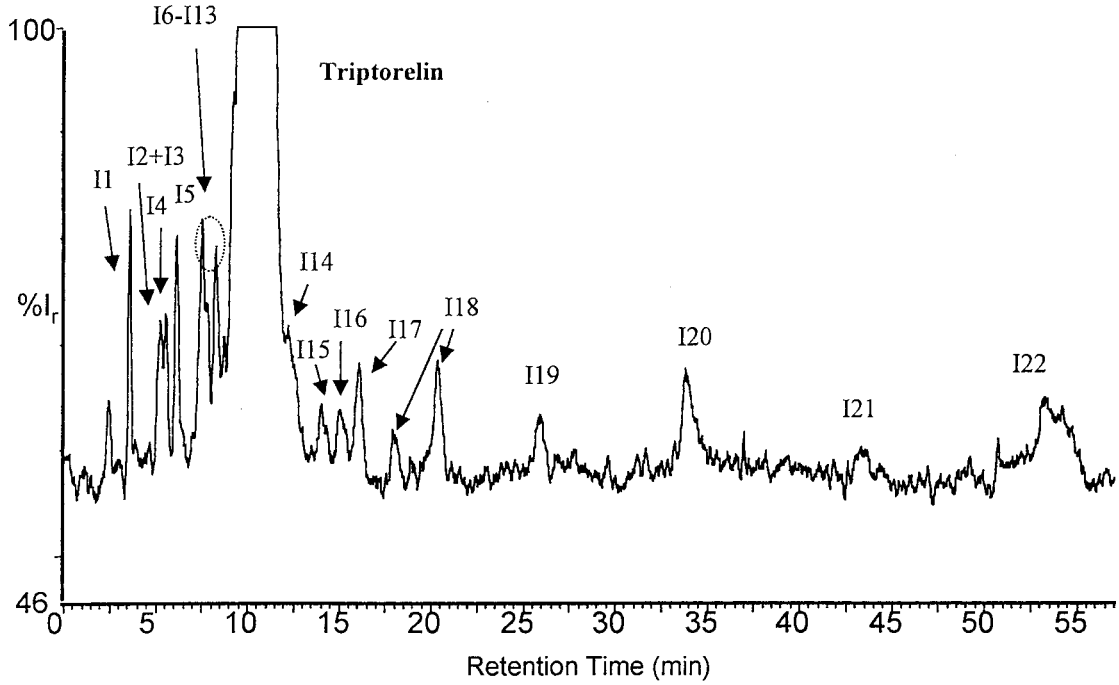


Figure 4. Total ion current (TIC) chromatogram of synthesis crude of peptide hormone triptorelin, with a mobile phase of (29:71)% (v/v) acetonitrile/water, 0.1% TFA.

In order to examine the accuracy of retention prediction using Eqn. (2), the selectivity α was obtained for adjacent solute pairs with separation problems, in the usual way:

$$\alpha = k_i/k_j \qquad (\text{where } k_i > k_j) \tag{3}$$

Figure 3 shows the variation in selectivity for the solute pairs vs. percentage of acetonitrile in the mobile phase. Only solutes whose separation was difficult were considered here. Solid lines indicate theoretical selectivity values obtained using Eqns (2) and (3) from only two measurements of k for each compound, and points represent experimental selectivity values. As shown, virtually the same values for selectivity are obtained from experimental data in the whole range of acetonitrile content and from estimations by Eqn. (2), indicating that only two experimental measurements per compound are enough to predict accurately the chromatographic behaviour and to optimize the mobile phase composition, in accordance with the results obtained in previous work.[11–15,34–37]

From Figs 2 and 3 we can conclude that the optimum chromatographic separation between the target peptide and the side products present in the crude can be achieved at percentages of acetonitrile in the mobile phase of 24–25% (v/v), Fig. 1. Thus, the mobile phase recommended for the purification of triptorelin using preparative chromatography on a C_8 column is 25% (v/v) acetonitrile/water, 0.1% TFA.

However, for reliable identification of the target peptide and associated side products using MS detection, an acetonitrile percentage of 29% (v/v) is preferred owing to the improvement of the analytical response at higher acetonitrile percentages. This mobile phase composition provides a good separation between almost all the substances present in the synthesis crude, allowing then identification by MS without confusing overlaps.

LC/ES-MS

Characterization of the synthesis crude of triptorelin

LC-ES-MS was performed under the conditions described in the Experimental section. The choice of a mobile phase with an acetonitrile percentage of 29% (v/v) provided a better signal than that obtained in more water-rich solvents, and allowed characterization of the low-abundance side products.[38,39]

The total ion current (TIC) chromatogram of the synthesis crude is shown in Fig. 4. The TIC chromatogram contains a major peak corresponding to the target peptide triptorelin, as well as a number of identified peaks from I1 to I22.

Figure 5 shows the spectra of the target peptide and the spectra associated with some of the chromatographic peaks I1, I2, I5, I6, I13, I17 and I19. The mass-to-charge ratios observed, the respective charged forms, and the average molecular masses estimated for each substance, as well as the proposed sequences for each ion, are summarized in Table 1. It can be observed in this table that some chromatographic peaks contain several co-eluting components. The identification of the co-eluting impurities was accomplished by using other acetonitrile percentages.

The method used to synthesize triptorelin provides useful information about the identify of the associated impurities.[40] Triptorelin was prepared by a SPPS method, following the Fmoc/*t*Bu (9-fluorenylmethoxycarbonyl/*tert*-butyl) strategy, which consists of successive coupling of all the amino acids in the desired sequence, with suitable protection, i.e., Fmoc group to protect the α-amino group, Pmc (2,2,5,7,8-

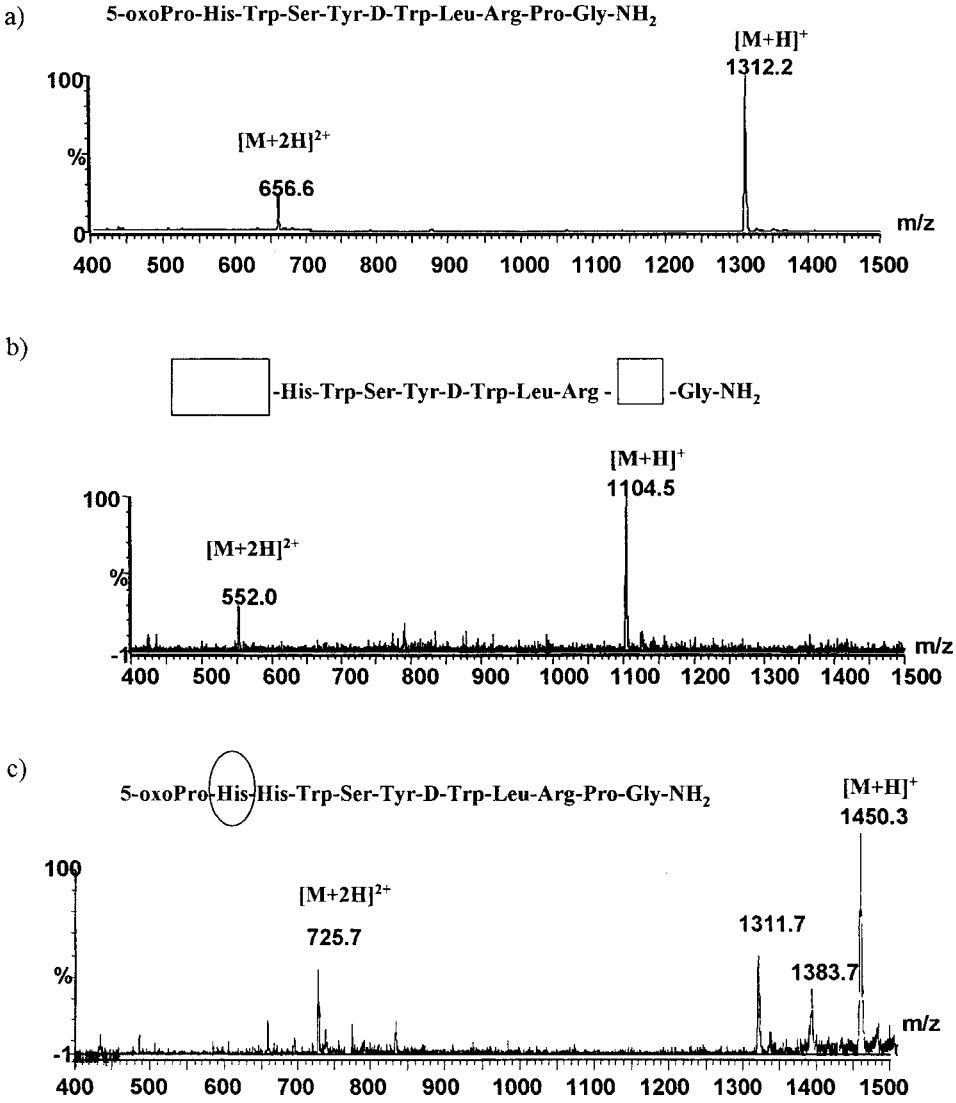


Figure 5. Electrospray mass spectra associated with the TIC chromatogram of Fig. 4 as well as the structures of the triptorelin and impurities that have been identified by LC/ES-MS. (a) Triptorelin, I2, I3, I7, I9, I10, I11 and I14; (b) $I19_2$; (c) $I5_1$, I6, I8, I12 and $I13_1$; (d) $I5_2$, I15, $I16_2$ and $I21_2$; (e) $I13_2$, $I16_1$, $I17_2$ and $I18_1$; (f) I1 and I4; and (g) $I17_1$, $I18_2$, $I19_1$, I20, $I21_1$, $I22_1$ and $I22_2$.

pentamethylcroman-6-sulfonyl) group to protect the arginine side chain, *t*Bu group to protect the tyrosine and serine side chains, and Trt (triphenylmethyl) group to protect histidine side chains.

1. Target peptide. The electrospray mass spectrum associated with the major peak in the TIC chromatogram at retention time 12.5 min (Fig. 5(a)) shows ions at *m/z* 656.6, corresponding to $[M+2H]^{2+}$, and at *m/z* 1312.2 corresponding to $[M+H]^{+}$. These ions indicate a molecular mass of 1311.2 Da in good agreement with the calculated molecular mass (isotope-averaged) of triptorelin of 1311.5 Da.

2. Impurities. Identification of these side products is based at present only on the experimental mass differences between the observed molecular mass of triptorelin and the molecular mass of each side product.

Racemization products

Racemization or isomerization of amino acids during solid phase peptide synthesis has been observed frequently,[41,42] mainly in the activation and coupling steps. The use of high concentrations of reagents to secure high coupling rates normally allows minimization of this undesired reaction. Racemic variants (diastereoisomers) of triptorelin have been identified in the chromatographic peaks I2, I3, I7, I9, I10, I11 and I14, for each of which the spectra contain ions at *m/z* 656.6 and 1312.2 associated with a molecular mass of 1311.2 ($\Delta M = +0.3$, defined as the deviation of the measured mass from the theoretical averaged molecular mass; Fig. 5(a)).

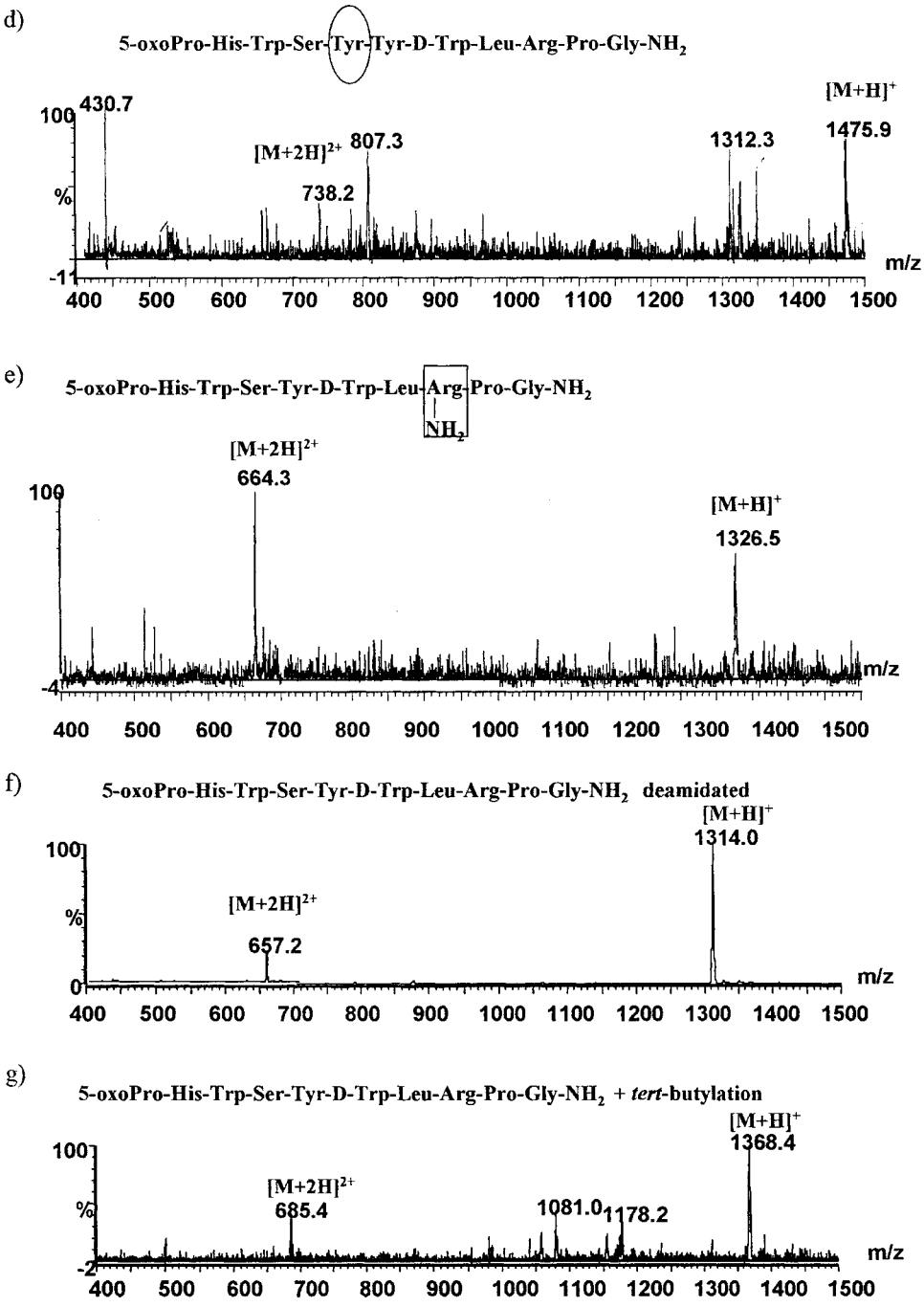


Figure 5. continued.

Deletion sequences

Imperfections in the removal of blocking groups can lead to the formation of chains from which one of the amino acid residues is absent. Such materials have been designated as 'failure sequences' or 'deletion sequences'.[39] This is the case for $I19_2$ (chromatographic peak I19 shows the coelution of two impurities). As an example, the spectrum of $I19_2$ is shown in Fig. 5(b) together with the proposed structure of this impurity. The spectrum of this impurity contains ions at *m/z* 552.0 and 1104.5, corresponding to a molecular mass of 1102.7 Da ($\Delta M = -208.4$), consistent with the absence of 5-oxoPro and Pro residues.

Amino acid insertions

The occasional insertion of an additional amino acid in the sequence can be produced by the use of an excess of equivalents in the coupling step to ensure the maximum efficiency. This is the case for impurities I5 ($I5_1$ and $I5_2$), I6, I8, I12, $I13_1$, I15, $I16_2$ and $I21_2$. The impurities $I5_1$, $I5_2$, I6, I8, I12 and $I13_1$ correspond to the insertion of an additional histidine (diastereoisomers), and the impurities $I15_2$, $I16_2$ and $I21_2$ correspond to the insertion of an additional tyrosine. Figures 5(c) and 5(d) show the associated spectra together with the structures of these by-products. In these spectra, additional ions can be observed (*m/z* 1383.7 and

Table 1. Results obtained by LC/ES-MS of the synthesis crude of triptorelin: mass-to-charge ratios (*m/z*) at retention time indicated (t_R), molecular masses (M), mass differences between the molecular mass of triptorelin and side product (ΔM) and sequence proposed

Chrom. peak	Substance	t_R (min)	*m/z* (Da/e)	M (Da)	ΔM	Sequence proposed
I1	I1	3.5	657.2 $[I1 + 2H]^{2+}$, 1314.0 $[I1 + H]^{+}$	1312.3	+1.1	Triptorelin deamidated
I2 + I3	I2	5.3	656.4 $[I2 + 2H]^{2+}$, 1312.8 $[I2 + H]^{+}$	1311.3	+0.1	Triptorelin diasteroisomer
	I3		656.7 $[I3 + 2H]^{2+}$, 1312.8 $[I3 + H]^{+}$	1311.6	+0.4	Triptorelin diasteroisomer
I4	I4	6.0	657.3 $[I4 + 2H]^{2+}$, 1312.8 $[I4 + H]^{+}$	1311.9	+0.8	Triptorelin deamidated
I5	$I5_1$	6.5	725.0 $[I5_1 + 2H]^{2+}$, 1450.5 $[I5_1 + H]^{+}$	1448.8	+137.5	Triptorelin + histidine
	$I5_2$		738.2 $[I5_1 + 2H]^{2+}$, 1475.9 $[I5_2 + H]^{+}$	1474.7	+163.5	Triptorelin + tyrosine
I6–I13	I6	8.4	725.3 $[I6 + 2H]^{2+}$, 1449.6 $[I6 + H]^{+}$	1448.6	+137.4	Triptorelin + histidine
	I7		656.5 $[I7 + 2H]^{2+}$, 1312.5 $[I7 + H]^{+}$	1311.3	+0.1	Triptorelin diasteroisomer
	I8		725.0 $[I8 + 2H]^{2+}$, 1450.3 $[I8 + H]^{+}$	1448.7	+137.5	Triptorelin + histidine
	I9		656.6 $[I9 + 2H]^{2+}$, 1312.2 $[I9 + H]^{+}$	1311.2	+0.0	Triptorelin diasteroisomer
	I10		656.6 $[I10 + 2H]^{2+}$, 1312.0 $[I10 + H]^{+}$	1311.1	−0.1	Triptorelin diasteroisomer
	I11		656.6 $[I11 + 2H]^{2+}$, 1312.3 $[I11 + H]^{+}$	1311.3	+0.1	Triptorelin diasteroisomer
	I12		725.2 $[I12 + 2H]^{2+}$, 1450.3 $[I12 + H]^{+}$	1448.8	+137.6	Triptorelin + histidine
	$I13_1$		725.0 $[I13_1 + 2H]^{2+}$, 1450.4 $[I13_1 + H]^{+}$	1448.7	+137.5	Triptorelin + histidine
	$I13_2$		664.3 $[I13_2 + 2H]^{2+}$, 1326.5 $[I13_2 + H]^{+}$	1326.1	+14.9	Addition of a NH_2 in the side chain of arginine
Triptore lin	Triptorelin	10.1	656.6 $[Tri + 2H]^{2+}$, 1312.2 $[Tri + H]^{+}$	1311.2	+0.0	Triptorelin
I14	I14	12.0	656.9 $[I14 + 2H]^{2+}$, 1312.4 $[I14 + H]^{+}$	1311.6	+0.4	Triptorelin diasteroisomer
I15	I15	14.1	738.2 $[I15 + 2H]^{2+}$, 1475.9 $[I15 + H]^{+}$	1474.7	+163.5	Triptorelin + tyrosine
I16	$I16_1$	14.9	664.3 $[I16_1 + 2H]^{2+}$, 1326.5 $[I16_1 + H]^{+}$	1326.1	+14.9	Addition of a NH_2 in the side chain of arginine
	$I16_2$		738.2 $[I16_2 + 2H]^{2+}$, 1475.9 $[I16_2 + H]^{+}$	1474.7	+163.5	Triptorelin + tyrosine
I17	$I17_1$	15.2	685.4 $[I17_1 + 2H]^{2+}$, 1368.2 $[I17_1 + H]^{+}$	1367.9	+56.7	Triptorelin + tButyl
	$I17_2$		664.0 $[I17_2 + 2H]^{2+}$, 1326.5 $[I17_2 + H]^{+}$	1325.8	+14.6	Addition of a NH_2 in the side chain of arginine
I18	$I18_1$	18.0	664.3 $[I16_1 + 2H]^{2+}$, 1326.5 $[I16_1 + H]^{+}$	1325.8	+14.6	Addition of a NH_2 in the side chain of arginine
	$I18_2$	20.1	685.0 $[I18 + 2H]^{2+}$, 1368.6 $[I18 + H]^{+}$	1367.8	+56.6	Triptorelin + tButyl
I19	$I19_1$	25.5	685.4 $[I19_1 + 2H]^{2+}$, 1368.3 $[I19_1 + H]^{+}$	1368.1	+56.9	Triptorelin + tButyl
	$I19_2$		552.0 $[I19_2 + 2H]^{2+}$, 1104.5 $[I19_2 + H]^{+}$	1102.7	−208.4	Triptorelin −(Pyr+Pro)
I20	I20	34.1	685.2 $[I20 + 2H]^{2+}$, 1368.6 $[I20 + H]^{+}$	1367.0	+55.8	Triptorelin + tButyl
I21	$I21_1$	43.5	684.7 $[I21_1 + 2H]^{2+}$, 1368.0 $[I21_1 + H]^{+}$	1367.2	+56.0	Triptorelin + tButyl
	$I21_2$		738.2 $[I21_2 + 2H]^{2+}$, 1475.9 $[I21_2 + H]^{+}$	1474.7	+163.5	Triptorelin + tyrosine
I22	$I22_1$	54.3	684.7 $[I22_1 + 2H]^{2+}$, 1368.4 $[I22_1 + H]^{+}$	1367.2	+56.0	Triptorelin + tButyl
	$I22_2$		685.4 $[I22_2 + 2H]^{2+}$, 1368.4 $[I22_2 + H]^{+}$	1367.1	+56.9	Triptorelin + tButyl

807.3), which are interpreted as degradation products because they are not observed at other acetonitrile percentages.

Impurities due to arginine side-chain reactions

Individual amino acid residues can undergo undesired reactions in their side chains, such as alkylations, oxidations, cyclizations, substitutions,etc.[43,44] This is the case for impurities $I13_2$, $I17_2$ and $I18_1$. The chromatographic peaks show similar mass spectra (Fig. 5(e)) that contain ions at *m/z* 664.3 and 1326.5, corresponding to a molecular mass of 1326.1 (ΔM = +14.9) which suggests the addition of a NH_2 group to the side chain of the arginine residue.

Deamidated impurities

Deamidation of the C-terminal amide side chain of an asparagine residue to form a free carboxylic acid may occur via either direct hydrolysis or via cyclic imide formation.[45] An increase of 1 Da in molecular mass with respect to that of the peptide, per deamidation site, can be measured from the corresponding spectra. This is the case for impurities I1 and I4. The chromatographic peaks contain side products which correspond to deamidated sequences. The spectra associated with these impurities contain ions at *m/z* 657.2 and 1314.0, corresponding to a molecular mass of 1312.3 (ΔM = +1.1), consistent with a change of one NH_2 group to an OH group (Fig. 5(f)).

tert-Butylated sequences

SPPS often yields partially protected impurities. In the deprotection steps a portion of blocked peptide chain is left fully protected after exposure to the deblocking reagent because the accessibility of some functional group is not always complete. This is the case for impurities $I17_1$, $I18_2$, $I19_1$, I20, $I21_1$ and I22 ($I22_1$ and $I22_2$). The chromatographic peaks contain side products which correspond to *tert*-butylated impurities. The spectra associated with these impurities contain ions at *m/z* 685.4 and 1368.4, corresponding to a molecular mass of 1368.1 Da (ΔM = +56.9; Fig. 5(g)).

CONCLUSIONS

The separation between the target peptide triptorelin and undesired side products in the synthesis crude has been optimized applying the LSER method. The present assessment of the analytical-scale conditions will facilitate pre-

parative-scale purification of the target peptide. Also, the data presented here demonstrate the capability of LC/ES-MS to furnish fast and reliable information on the various products of the solid phase synthesis of peptides. The characterization of side products helps fulfil the necessary requirements for triptorelin commercialization as a peptide of therapeutic interest. In addition, it will improve the process of synthesis (SPPS) by suitable modification of those steps in which potential side products are produced, thus improving the purity of the crude by decreasing the formation of the above-mentioned impurities.

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