ELSEVIER

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Fractionation and characterization of a crude peptide mixture from the synthesis of eledoisin by liquid chromatography–electrospray ionization mass spectrometry

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Abstract

Eledoisin is a peptide with complex pharmacological properties and recognized therapeutical application. Peptides of pharmaceutical interest in the molecular mass range of eledoisin are often synthesized using solid-phase peptide synthesis. This procedure results in complex crudes of reaction that require extensive purification and characterization. In order to avoid long empirical optimization procedures to separate the unwanted products associated with the target peptide eledoisin from a synthesis crude, the method of linear solvation energy relationships for predicting peptide retention times was applied and the proportion of the organic modifier of the mobile phase used in high-performance liquid chromatography (HPLC) method was optimized. The crude of synthesis was also analyzed by combined HPLC–electrospray ionization mass spectrometry, which allowed rapid and reliable identification of the target peptide and has furnished comprehensive information on other reaction products.

Keywords: Linear solvation energy relationships; Solid-phase peptide synthesis; Electrospray ionization; Peptides; Eledoisin

1. Introduction

Eledoisin is a member of the tachynin family of peptides with complex pharmacological properties, including being a powerful vasodilator, having hypotensive action and the stimulation of extravascular smooth muscle. Almost 20 years ago Bietti and coworkers first reported the successful treatment of dry eyes with eledoisin, an endecapeptide that can be isolated from the salivary glands of a mediterranean octopus, *Eledona mashata* [1–4].

Most of the present day peptides of pharmaceutical interest in the molecular mass range 500–8000, like eledoisin, are often synthesized using solid-phase peptide synthesis (SPPS) [5]. The procedure commonly results in complex crudes of reaction, which are mainly peptide chains containing a fewer amino acids than those present in the target peptide, as well as by-products associated, for example, with oxidation, *tert*.-butylation, and racemization of some amino acid [6].

Pharmaceutical products require extensive purification and characterization in order to be commercialized, not only of the peptide itself but also of the impurities resulting from the process steps [7]. Taking into account the complexity of synthesis crudes, the preparation of significant amounts of

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purified peptides requires, firstly, assessment of specific analytical conditions. In order to avoid long empirical optimization procedures to separate the unwanted products associated with the peptide eledoisin from a crude of synthesis, a system for predicting peptide retention times is of great advantage. In this study, the proportion of the organic modifier of the mobile phase was optimized by establishing relationships between retention and Reichardt's E_T^N scale of solvent polarity, using the method of linear solvation energy relationships (LSERs), as used in previous studies [8–11].

Characterization of side products associated with the target peptide is desirable in order to design and improve the synthesis procedures of peptides and is also needed for the commercialization of the peptide product. Mass spectrometry is now considered to be a significant aid in peptide and protein identification [12,13]. In this study electrospray ionization mass spectrometry (ESI-MS) has been used successfully for the characterization of synthesis crude of eledoisin. The combination of these two powerful analytical techniques, HPLC and ESI-MS, has allowed rapid and reliable identification of the target peptide and has furnished comprehensive information on other reaction products [13–16].

2. Experimental

2.1. Chemicals and reagents

Water with a conductivity lower than 0.05 μS/cm and acetonitrile (ACN; Merck, Darmstadt, Germany) were of HPLC grade. Trifluoroacetic acid (TFA), potassium bromide and potassium hydrogenphthalate were all analytical grade obtained from Merck. The synthesis crudes of peptide eledoisin used in this study were purchased from Lipotec (Barcelona, Spain). The sequence of eledoisin is: pGlu–Pro–Ser–Lys–Asp–Ala–Phe–Ile–Gly–Leu–Met–NH_2. The synthesis crudes were stored in a freezer at 0°C when not in use. Working solutions of crude of synthesis were prepared at concentrations of 1 and 3 mg/ml for HPLC–UV analysis and HPLC–ESI-MS analysis respectively, using the mobile phase as solvent.

2.2. Apparatus

2.2.1. HPLC–UV

The chromatographic equipment consisted of an Isco Model 2350 (Lincoln, NE, USA) pump with an injection valve with a 100 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) running on a personal computer. A Kromasil C_8 (5 μm) column 250×4.6 mm I.D. 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, Barcelona, Spain) using an Orion 8102 ROSS combination pH electrode (Orion, Boston, MA, USA). The potentiometric system was calibrated using a standard reference solution of potassium hydrogenphthalate 0.05 mol/kg whose reference pH values in the acetonitrile–water mixtures studied were assigned previously [17–20].

2.2.2. HPLC–ESI-MS

HPLC–ESI-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 50 μl sample loop, coupled to a VG Platform II single quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a nebulizer-assisted electrospray source.

The optimal operating conditions were: drying nitrogen gas flow (300–400 L/h); ESI nebulizing nitrogen gas flow (10–20 L/h); electrospray probe (capillary) voltage (3.5 kV); counter electrode (HV lens) voltage (0.5 kV); sample cone voltage (25 V); source temperature (80°C). All spectra were obtained in full scan mode in the range of m/z 200–1300.

The separation was performed on a LiChrospher 100 RP-18 (5 μm) column 250×4 mm I.D., at room temperature and with a post-column split 1/50.

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.

2.3. Chromatographic procedure

2.3.1. HPLC–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) trifluoroacetic acid, with pH of mobile phase 1.9–2.0, at several concentrations of acetonitrile from 24–28% (v/v) [21,22]. 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 bromide solution [0.01% (w/v) in water] and monitoring the signal at 200 nm [23]. The retention times and the capacity factors of the solutes were determined from three injections of 1 mg/ml solution of eledoisin 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 takes place.

2.3.2. HPLC–MS procedure

For identification of different side products of the crude of synthesis, 3 mg/ml solution of eledoisin crude was injected into the HPLC–ESI-MS system. The LiChrospher C_{18} column was equilibrated for 40 min with the selected mobile phase of acetonitrile–water (27.5:72.5, v/v) containing 0.1% (v/v) TFA, of which the pH of mobile phase is 1.9–2.0. ESI spectra were obtained at optimum conditions of the mass spectrometer shown in the Experimental section [24] and working in full scan mode (m/z 200–1300), of target peptide eledosin and of the associated side products.

3. Results and discussion

3.1. HPLC–UV

The synthesis crude of eledoisin was examined by analytical HPLC. The resulting UV chromatogram contains a major peak, the target peptide (eledoisin), as well as a number of peaks corresponding to unidentified peptides A, B, C, D, E and F (Fig. 1).

The logarithm of the capacity factor values (log k') were obtained at different percentages of acetonitrile from 24–28% (v/v) for the target peptide and for the associated side products (Table 1). Mobile phases assayed were acetonitrile–water mixtures in the volume ratios (24:76), (25:75), (26:74) and (28:72).

To optimize the composition of the mobile phase, the Reichardt's E_T^N polarity parameter (known for the whole range of composition acetonitrile–water) [25] (Table 1), was related with the capacity factors of the target peptide and associated by-products to be separated, using the LSER methodology.

The LSER approach, when applied to chromatographic processes, correlates a general solute property (SP) such as logarithmic capacity factor, with feature parameters of solute and both mobile and stationary phases [26]. This correlation can be expressed as follows in a system with a fixed pair of solute and stationary phase [8,9]:

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

where $(\log k')_0$ depends on the parameters of the stationary phase and the solute, s, a and b are the correlation coefficients which depend also on the solute and stationary phase parameters, and π^*, α and β are the Kamlet–Taft solvatochromic parameters of the mobile phase which are known over the whole range [27,28] of composition studied and evaluate solvent polarizability/dipolarity (π^*) and solvent hydrogen-bond acidity (α) and basicity (β) respectively.

The difference in β values between water and acetonitrile is small [27,28]. Moreover, β values are constant over most of the composition range. Therefore, the β term in Eq. (1) can be included in the independent term. Thus, taking into account the observed correlation: $E_T^N = 0.009 + 0.415\pi^* + 0.465\alpha$ [29], Eq. (1) can be reduced to Eq. (2),

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

as demonstrated in previous studies [8–11] for analytes with very different structural characteristics, which expresses a linear relationship between the chromatographic capacity factor values and the normalized E_T^N scale of the mobile phase polarity

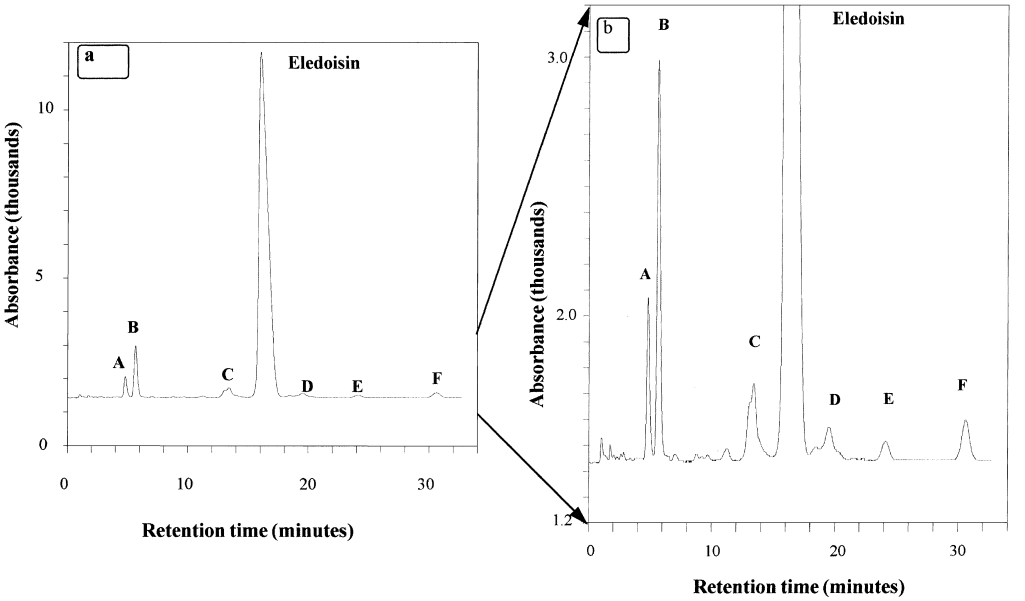

Fig. 1. (a) UV chromatogram of a solution containing 1 mg/ml of crude of synthesis of peptide eledoisin, with a mobile phase of acetonitrile–water (26:74, v/v), 0.1% TFA. (b) Expansion on scale *y*-axis of UV chromatogram.

[30]. Therefore, suitable prediction of the retention for a specific solute can be achieved from the E_T^N values of the mobile phase and a few experimental data. In this case, only three experimental data for each substance studied were used to correlate log k' versus E_T^N values of ACN–water systems (Fig. 2) corresponding to the mixtures with percentages of 25, 26 and 28% (v/v) (correlation coefficients greater than 0.99).

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

$$\alpha = k'_j / k'_i. \quad (3)$$

Fig. 3 shows variation of selectivity for the solute pairs versus percentage of acetonitrile in the mobile phase. Solid lines indicate theoretical selectivity values obtained using Eq. (3), and points represent experimental selectivity values. Fig. 3 shows that values of α are concordant from the two methods. Thus it appears clear that two measurements per compound of k' are enough to predict the optimum mobile phase composition with accuracy, in accordance with the results obtained in previous works [8–11].

Table 1
Logarithms of the capacity factor values of the eledoisin and impurities, and the E_T^N parameter values at various percentages of acetonitrile in the mobile phase

ACN (%)	E_T^N	Log k'						
		Impurity A	Impurity B	Impurity C	Eledoisin	Impurity D	Impurity E	Impurity F
24	0.877	0.353	0.544	1.065	1.142	1.223	1.322	1.351
25	0.876	0.283	0.365	0.858	0.926	1.012	1.118	1.157
26	0.872	0.218	0.295	0.784	0.845	0.936	1.040	1.079
28	0.864	0.015	0.079	0.563	0.615	0.705	0.805	0.845

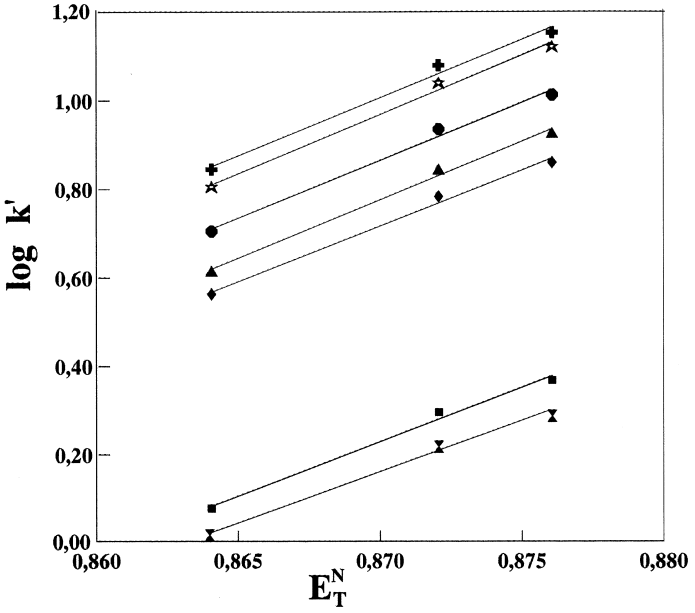

Fig. 2. Plots of log k' of eledoisin and impurities versus E_T^N parameters of the mobile phase: Impurity A (⧗), impurity B (■), Impurity C (♦), Eledoisin (▲), Impurity D (●), Impurity E (★), Impurity F (✚).

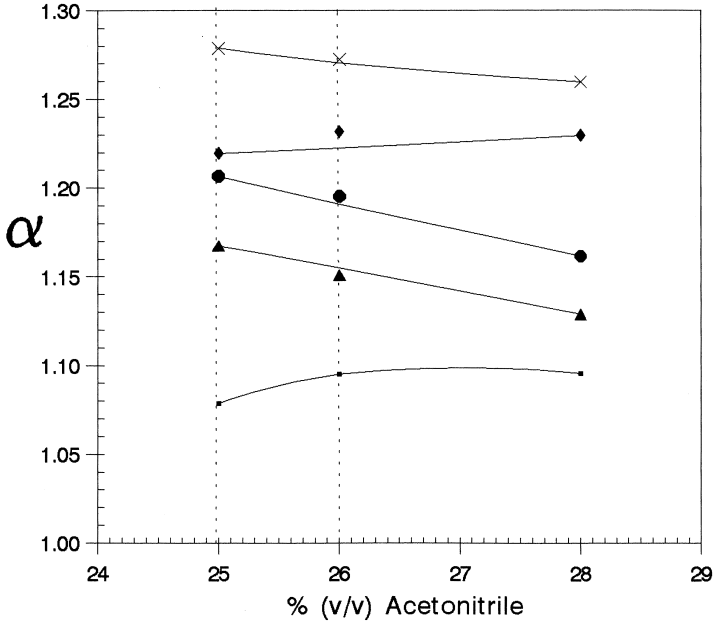

Fig. 3. Variation in selectivity values for compound pairs with acetonitrile percentage. Solid lines indicate predicted values of selectivity from Eq. 2 and points represent experimental values of selectivity: Impurity B/Impurity A (●), Eledoisin/Impurity C (▲), Impurity D/Eledoisin (♦), Impurity E/Impurity D (✖), Impurity F/Impurity E (■).

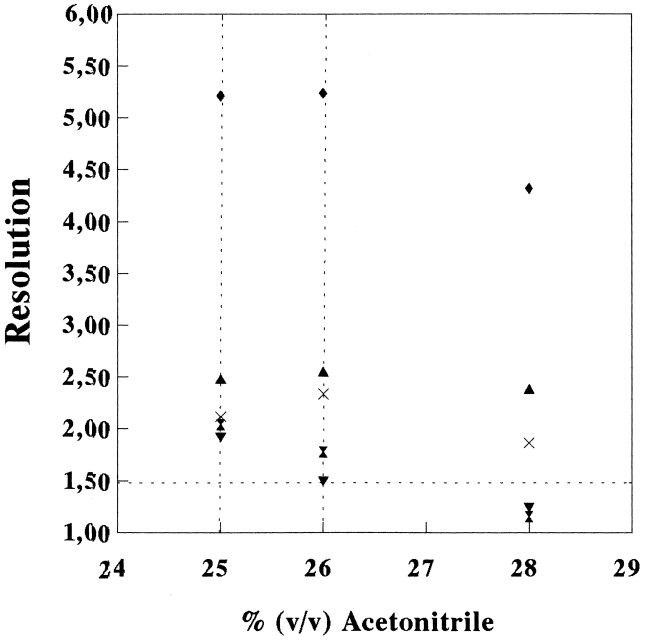

Fig. 4. Variation in experimental resolution for compound pairs with acetonitrile percentage. Impurity B/Impurity A (⧗), Eledoisin/Impurity C (▼), Impurity D/Eledoisin (▲), Impurity E/Impurity D (♦), Impurity F/Impurity E (✖).

Likewise, the main goal of practicing chromatographer is the resolution of all the analytes of interest in a given sample. Fig. 4 shows variation of experimental resolution (R_s) for the solute pairs versus percentage of acetonitrile in the mobile phase, obtained from the typical relation $R_s = 2(t_{R1} - t_{R2})/(w_2 - w_1)$.

From Figs. 2–4 we can concluded 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 25–26% (v/v). The chromatogram of the crude of synthesis of eledoisin using UV detection obtained from injection of 1 mg/ml solution at optimal conditions is shown in Fig. 1. Thus, the mobile phase recommended for the purification of eledoisin using preparative chromatography is 25% (v/v) acetonitrile in water, 0.1% TFA; percentages of acetonitrile $<25\%$ (v/v) present longer analysis times.

3.2. HPLC–MS

Operating parameters have been optimized in a previous study [24], with the exception of the sample cone voltage which has been varied from the initial value suggested, to reduce the fragmentation produced a higher cone voltages.

It can be observed that at lower cone voltages ESI yields simple mass spectra with negligible fragmentation allowing reasonable identification of the molecular identities, which is essential for characterization of impurities. For this reason, spectra were obtained using sample cone voltage of 25 V. Optimal conditions are shown in experimental section.

The total ion current (TIC) chromatogram of the crude of synthesis is shown in Fig. 5. Like the UV chromatogram the TIC contains, a major peak corresponding to the target peptide eledosin, as well as a number of identified peptides peaks A, B, D, E and F (impurity C was not identified due to co-elution with the eledoisin major peak). Fig. 6 shows the spectrum of the target peptide and Fig. 7 shows the spectra of some selected impurities associated with peaks A, D, E and F, and also the spectra of some selected impurities that coelute with the major peak: impurities 1 and 2.

The chromatograms of Figs. 1 and 5 were gener-

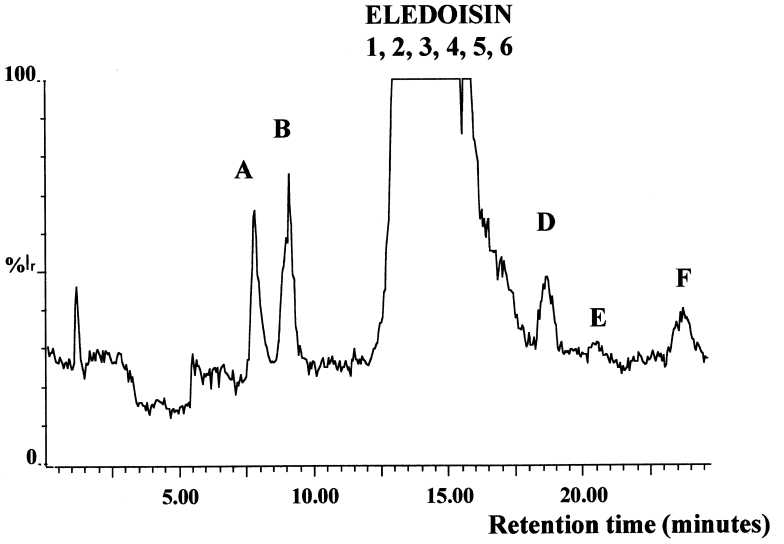

Fig. 5. Total ion current (TIC) chromatogram of a solution containing 3 mg/ml of the crude of synthesis of peptide eledoisin, with a mobile phase of acetonitrile–water (27.5:72.5, v/v), 0.1% TFA.

ated in different experimental conditions including different columns and different sample concentrations. With HPLC–MS we have selected the LiChrospher instead of Kromasil column for these studies due to better response of LiChrospher-MS system; for this reason, we have lightly changed the mobile phase composition.

It is important to consider the method of synthesis in order to identify the target peptide and most associated impurities [31]. Eledoisin was prepared by Lipotec, applying linear SPPS, following the Fmoc/*t*Bu (9-fluorenylmethoxycarbonyl/*tert.*-butyl) strategy, which consisted in coupling one to one all the amino acids contained on the sequence with suitable protection except the last which is pyroglutamic acid and does not need protection. After construction of the complete protected sequence on the resin, a subsequent cleavage of the peptide–resin bond and deprotection of the other side chains were carried out by acidolytic treatment (TFA), obtaining the final product eledoisin.

Table 2 summarizes the m/z values observed with the relative intensities of the relevant peaks of the mass spectra in parenthesis, the structural assignments of respective charged forms, the molecular mass estimated for each substance as well as mass differences between molecular mass of eledoisin and side products. The molecular mass values, *M*, were calculated using an averaging algorithm of MassLynx software, from the set of m/z peaks of spectrum corresponding to the series of charged states of each molecular species.

3.2.1. Target peptide

The electrospray mass spectrum associated with the major peak TIC in the chromatogram, at retention time, $t_R = 13.5$ min, Fig. 6, shows at m/z 1189.0, corresponding to the $[M+H]^+$ and m/z 595.2 corresponding to the $[M+2H]^{2+}$. These ions indicate a molecular mass of 1188.2, which is in good agreement with the calculated value of 1188.4. The ion at m/z 586.8 yielding the molecular mass of 1171.6 is attributed to the loss of neutral group ammonia from eledoisin. It is not known whether this loss of ammonia was produced by the electrospray process or whether it is due to an impurity in the sample. Likewise, the m/z values of ions shown in Fig. 6, are attributed to several fragmentations of eledoisin and their interpretation is based on the nomenclature presented by Biemann [32]. The structure of eledoisin and the scheme of its fragmentations are shown in Fig. 6.

The spectra of some of the impurities detected in the crude of synthesis are shown in Fig. 7 and the

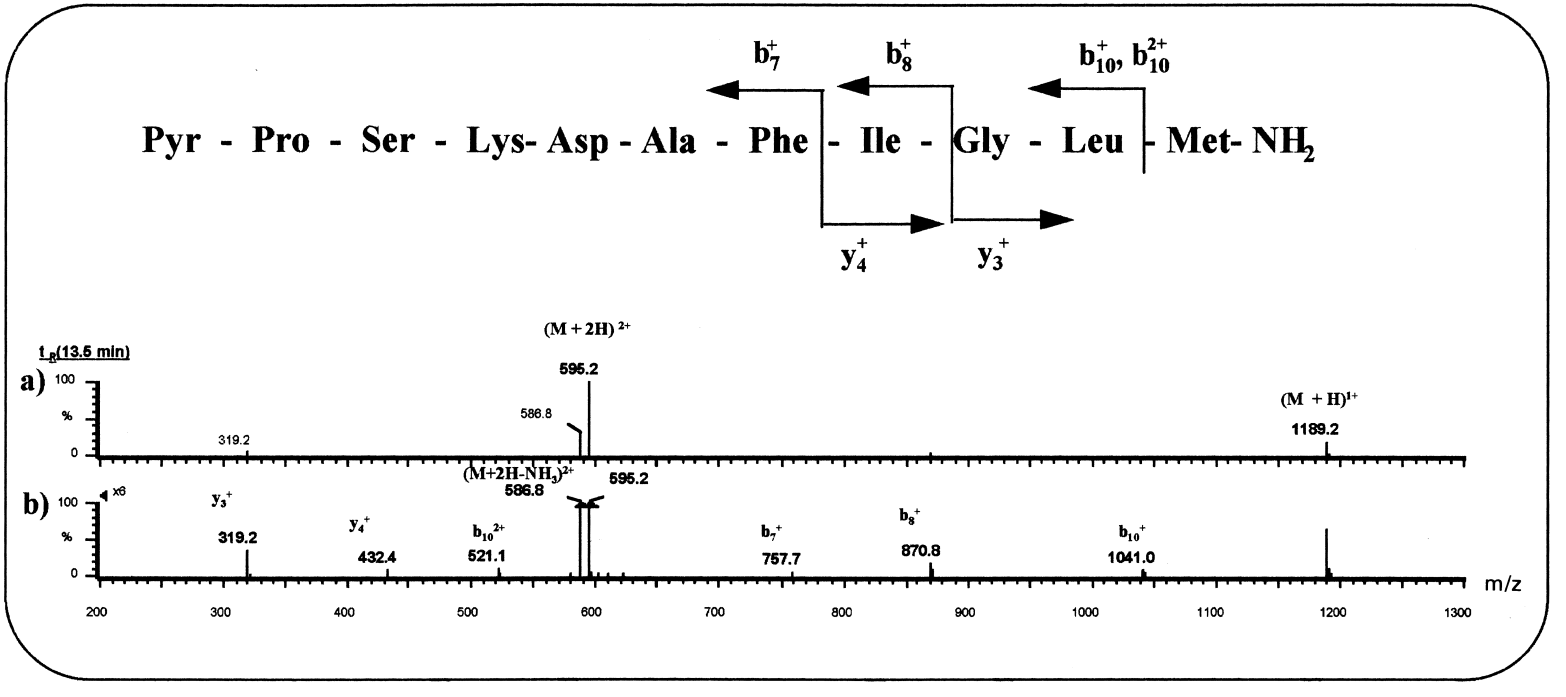

Fig. 6. Structures of eledoisin and fragments that have been identified by HPLC–ESI-MS, and the electrospray mass spectrum of eledoisin as well as its expansion on *y*-axis.

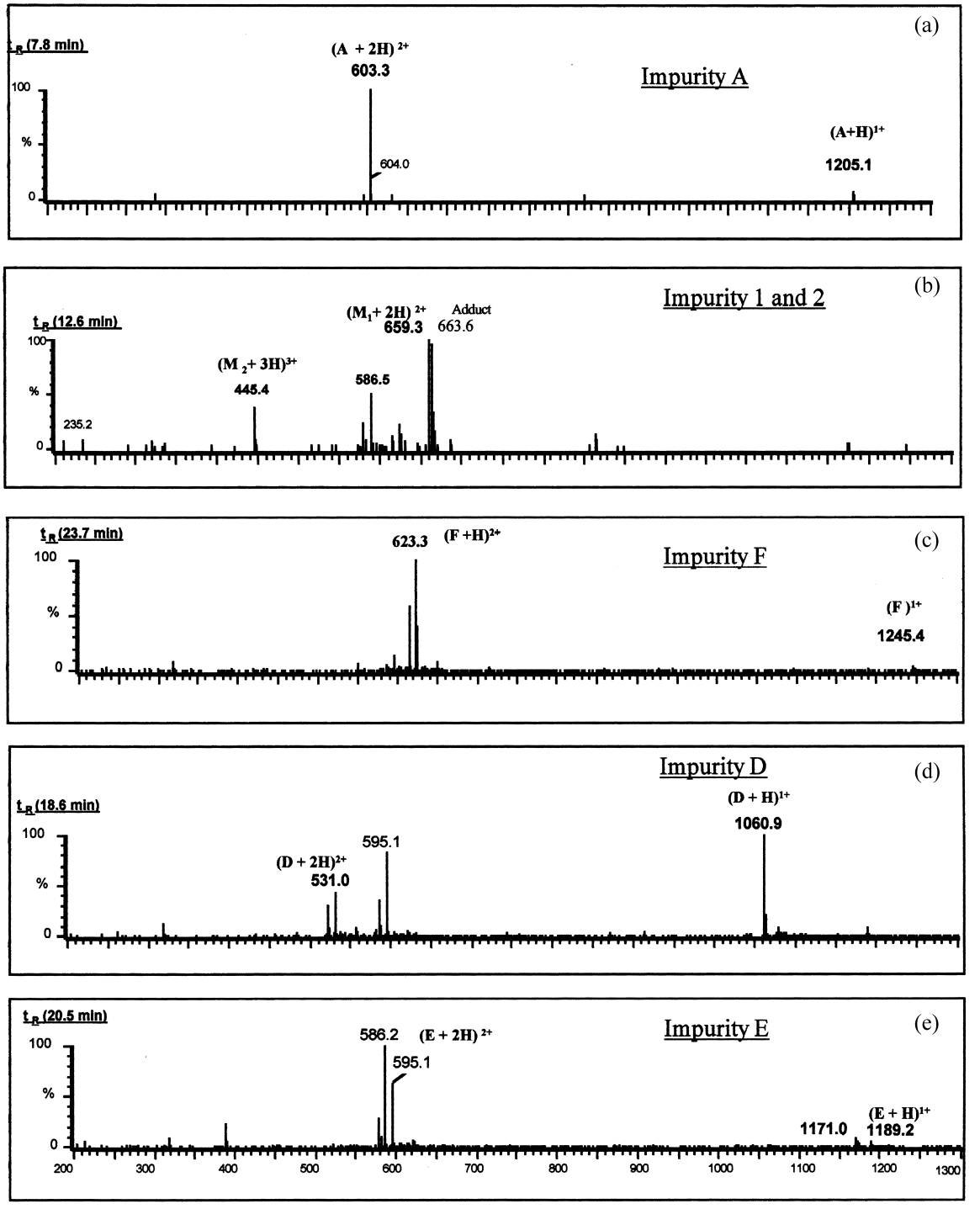

Fig. 7. Electrospray mass spectra associated with the TIC peaks at the retention time indicated: (a) 7.8 min, (b) 12.6 min, (c) 23.7 min, (d) 18.6 min, and (e) 20.5 min.

Table 2
Results obtained by HPLC–ESI-MS of the crude of synthesis of eledoisin: mass-to-charge ratios (m/z) with relative intensities in parenthesis, structural assignments, at retention time indicated (t_R), molecular masses (M) and mass differences between the molecular mass of eledoisin and side product (ΔM)

Substance	t_R (min)	m/z	M	ΔM
Eledoisin	13.5	595.2 (55.0) $[M+2H]^{2+}$, 1189.0 (9.4) $[M+H]^{+}$ (Fragments: 586.8 (16.3) $[M-NH_3+2H]^{2+}$, 870.8 (1.3) $[M-GlyLeuMetNH_2+H]^{+}$, 319.2 (3.0) $[GlyLeuMetNH_2+H]^{+}$, 757.7 (0.4) $[M-IleGlyLeuMetNH_2+H]^{+}$, 432.4 (0.7) $[IleGlyLeuMetNH_2+H]^{+}$, 1040.9 (0.7) $[M-MetNH_2+H]^{+}$, 521.0 (0.5) $[M-MetNH_2+2H]^{2+}$)	1188.2	−0.0
Impurity A	7.8	603.3 (50.2) $[A+2H]^{2+}$, 1205.1 (2.8) $[A+H]^{+}$	1204.4	+16.2
Impurity B_1	9.1	623.3 (37.2) $[B_1+H]^{2+}$, 1245.1 (1.1) $[B_1]^{+}$	1245.4	+57.2
Impurity B_2	9.1	595.2 (13.6) $[B_2+2H]^{2+}$ (Fragments: 586.8 (2.4) $[B_2-NH_3+2H]^{2+}$, 571.1 (3.8) $[B_2-SHCH_{3+}+2H]^{2+}$)	1188.4	+0.2
Impurity 1	12.6	659.2 (11.0) $[M_1+2H]^{2+}$ (Adduct: 663.6 (10.5) $[M_1+1/2H_2O+2H]^{2+}$)	1316.4	+128.2
Impurity 2	12.6	445.4 (4.2) $[M_2+3H]^{3+}$	1333.2	+145.0
Impurity 3	12.8	623.5 (11.0) $[M_3+H]^{2+}$, 1246.0 (0.8) $[M_3]^{+}$	1245.9	+57.7
Impurity F	23.7	623.3 (23.5) $[F+H]^{2+}$, 1245.4 (0.9) $[F]^{+}$	1245.5	+57.3
Impurity 4	12.9	546.7 (6.4) $[M_4+2H]^{2+}$, 1091.8 (1.4) $[M_4+H]^{+}$	1091.1	−97.1
Impurity 5	12.9	539.5 (4.7) $[M_5+2H]^{2+}$	1077.0	−111.2
Impurity 6	16.6	551.6 (8.0) $[M_6+2H]^{2+}$, 1102.0 (1.2) $[M_6+H]^{+}$	1101.1	−87.2
Impurity D	18.7	531.0 (2.3) $[D+2H]^{2+}$, 1060.9 (15.3) $[D+H]^{+}$	1060.0	−128.2
Impurity E	20.5	595.1 (12.3) $[E+2H]^{2+}$, 1189.2 (1.1) $[E+H]^{+}$ (Fragments: 586.2 (19.7) $[E-H_2O+2H]^{2+}$, 1171.0 (1.7) $[E-H_2O+H]^{+}$)	1188.2	0.0

structure of every detected impurity is shown in Fig. 8. Identification of these side products is based only on the experimental mass differences between the observed molecular mass of eledosin, 1188.2 and the molecular mass of each side product.

3.2.2. Impurity A

Fig. 7a shows the spectrum associated with the chromatographic peak A ($t_R = 7.8$ min) which contains an ion at m/z 603.3 corresponding to the $[A+2H]^{2+}$ ion at m/z 1205.1 corresponding to the

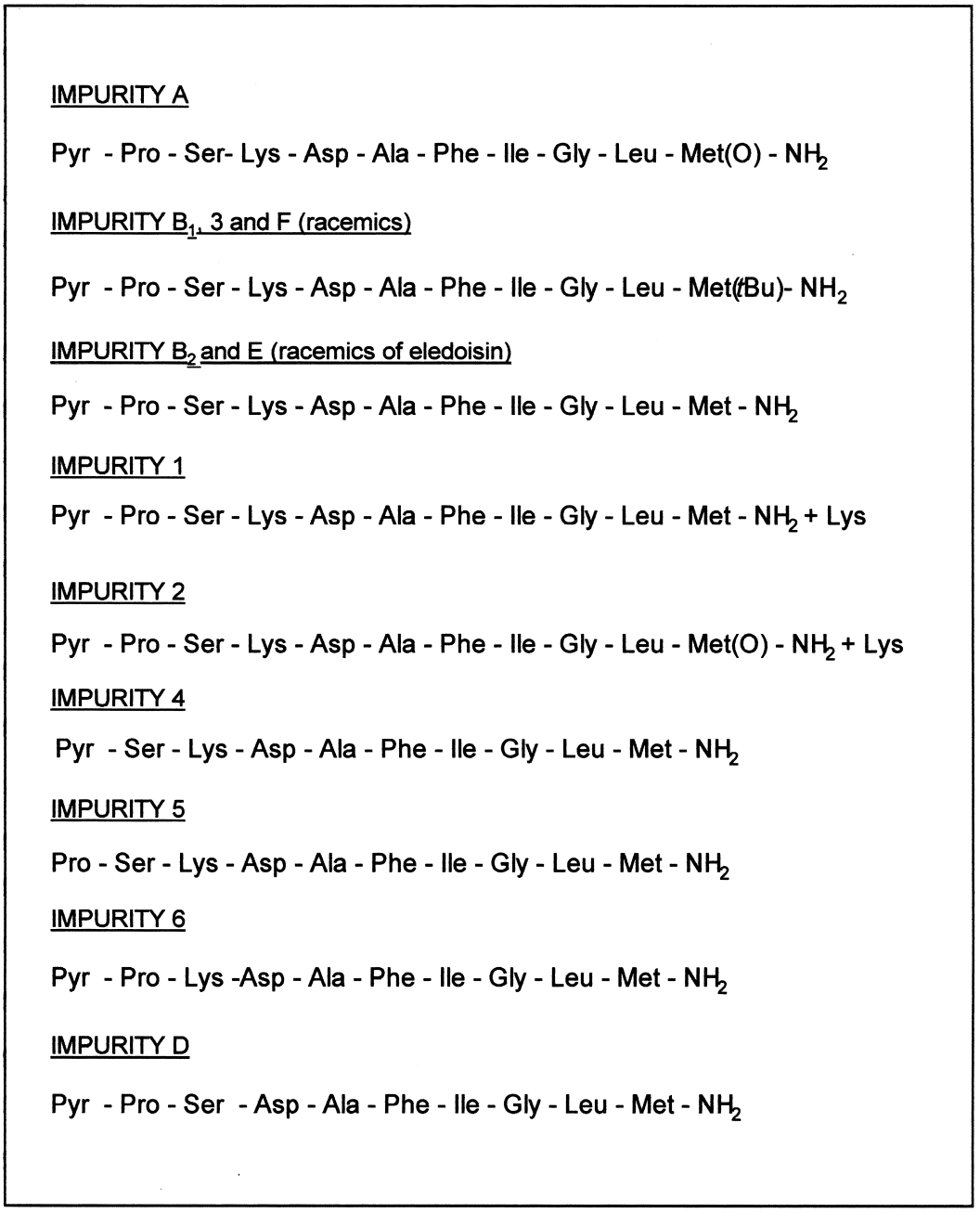

Fig. 8. Proposed structures of impurities of crude of synthesis of eledoisin that have been identified by HPLC–ESI-MS.

$[A+H]^+$ ion. These ions are associated with a molecular mass of 1204.4 (difference of mass respect to eledoisin $\Delta M = +16.2$). The major side reactions associated with residue of methionine, are alkylation and oxidation of the thioether side chain, being oxidation to sulfoxides formed simply on prolonged exposure to air [6]. For this reason this molecular mass is attributed to an impurity with a similar sequence to eledoisin and one oxidation in the thioether group of residue of methionine, leading to the formation of [Met(O)^{11}] eledoisin.

3.2.3. Impurities B_1 and B_2

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. The spectrum of peak B, Table 2, shows some complexity due to the coelution of two impurities at $t_R = 9.1$ min; the first, B_1, presents a double-charge state distribution ion at m/z 623.3 corresponding to the $[B_1+H]^{2+}$ double charge state and the ion at m/z 1245.1 corresponding to the $[B_1]^+$ single charge state, yielding the molecular mass of 1245.4. This mass could be attributed to an impurity with a similar sequence to eledoisin and one *tert.*-butylation in the methionine residue, leading to [Met(*t*Bu)^{11}] eledoisin. The methionine *tert.*-butylation does not often occur if the thioether group is protected, although it may become so under several circumstances [6]. The second co-eluting substance, B_2, with the m/z of 595.2 corresponding to the $[B_2+2H]^{2+}$ double charge state which gives a value of molecular mass of 1188.4, attributed to a racemic peptide of eledoisin. The m/z 586.8 and 571.1 are interpreted as fragmentation products corresponding, respectively, to loss of ammonia $[B_2-NH_3+2H]^{2+}$, and methanethiol groups $[B_2-HSCH_3+2H]^{2+}$ from racemic eledoisin, which may have been tentatively formed in the ionization source.

3.2.4. Impurities 1 and 2

The spectrum associated with peak 1 ($t_R = 12.6$ min) (Fig. 7b) exhibits m/z of 659.2 $[M_1+2H]^{2+}$, yielding the molecular mass of 1316.6. The difference between its molecular mass and the mass of eledoisin is +128.2 corresponding to the lysine residue. This difference indicates the presence of a modified eledoisin chain due to the addition of lysine at an unknown position, leading to *endo*-Lys-eledoisin. In this spectrum, we can also see the m/z of 663.6, corresponding to an adduct of the latter with half a water molecule.

At the same time of retention $t_R = 12.6$ min, takes place the coelution of impurity 2 (Fig. 7b), with a m/z value of 445.4, $[M_2+3H]^{3+}$, yielding the molecular mass of 1333.2, attributed to oxidized *endo*-Lys-eledoisin in the thioether group of the residue of methionine which correspond to *endo*-Lys-[Met(O)^{11}] eledoisin..

3.2.5. Impurities 3 and F

The side products associated with the chromatographic peaks at $t_R = 12.8$ min, impurity 3, and $t_R = 23.7$ min, impurity F, Table 2 and Fig. 7c respectively, have also been attributed to racemics of the impurity B_1, [Met(*t*Bu)^{11}] eledoisin, because their spectra contain similar mass-to-charge ratios.

3.2.6. Deletion sequences: impurities 4, 5, 6, D and E

Imperfections in the removal of the blocking group lead to the formation of chains from which one of the amino acid residues is absent, designated as deletion sequences. In this way, impurities 4, 5, 6, D and E are considered deletion sequences.

3.2.6.1. Impurities 4 and 5. The spectrum associated with impurity 4 ($t_R = 12.9$ min), Table 2, shows the ratios m/z 546.7, $[M_4+2H]^{2+}$, and m/z 1091.8, $[M_4+H]^+$, yielding the molecular mass of 1091.1 (difference of mass with respect to eledoisin −97.1), which corresponds to the deletion of the proline residue, des-Pro^2-eledoisin. In this spectrum we can also see the ion at m/z 539.5, $[M_5+2H]^{2+}$, yielding the molecular mass of 1077.0 (difference of mass with respect to eledoisin −111.2), which corresponds to the deletion of the pyroglutamic acid residue at the N-terminal position, des-Pyr^1-eledoisin, impurity 5.

3.2.6.2. Impurity 6. Impurity 6, with associated spectrum at $t_R = 16.6$ min, table 2, shows the ratios m/z 551.6 $[M_6+2H]^{2+}$, and m/z 1102.0, $[M_6+H]^+$

yielding the molecular mass of 1101.1. This latter fragment differs from the former in −87.1, which implies the deletion of the serine residue, des-Ser^3-eledoisin.

3.2.6.3. Impurity D. Likewise, impurity D, with associated spectrum at $t_R = 18.6$ min (Fig. 7d), exhibits two charge state ratios corresponding to m/z 531.0, $[D+2H]^{2+}$, and m/z 1060.9, $[D+H]^{+}$, yielding the molecular mass of 1060.0. This latter fragment differs from the former by −128.3, which implies the loss of the lysine residue, des-Lys^4-eledoisin.

3.2.6.4. Impurity E. In the same way, the spectrum associated with impurity E ($t_R = 20.5$ min) (Fig. 7e), contains two charge state ratios corresponding to m/z 595.1, $[E+2H]^{2+}$, and m/z 1189.2, $[E+H]^{+}$, yielding the molecular mass of 1188.2. This latter impurity could be tentatively considered as a racemic of eledoisin. This spectrum shows also two charge state ratios corresponding to m/z 586.2 and m/z 1171.0 yielding the mass molecular of 1170.2, that differs from impurity E in −18.0, which implies the loss of a water molecule.

This procedure for the synthesis of eledoisin appears to have resulted in oxidized peptide species and *tert*.-butylated peptide species due to the presence of a methionine residue, racemic peptide species, an inherent problem in SPPS that may occur at several steps of synthesis, deletion peptide sequences species due to the deletion of some amino acid and modified sequence peptide species due to the insertion of some amino acid. The presence of these impurities in the crude of synthesis hinders purification, since the properties of such species are generally similar to those of the target peptide. The results obtained demonstrate that the HPLC–ESI-MS system provides an efficient analytical tool for fast separation and reliable identification of the various products of synthesis crudes, the target peptide and the associated impurities.

Furthermore, the characterization of by-products of synthesis may, first, fulfil the necessary requirements for eledoisin commercialization as a peptide of therapeutic interest; second, improve the process of synthesis (SPPS) by suitable modification of those steps in which potentially by-products are produced and third, improve the purity of the crude decreasing the formation of above-mentioned impurities and, therefore, their presence in the crude of synthesis.

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