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Short communication

Investigation of crudes of synthesis of carbetocin by liquid chromatography coupled to electrospray ionization mass spectrometry[1]

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Abstract

Synthetic peptides are becoming increasingly important as drugs in the pharmaceutical industry. Stepwise solid-phase peptide synthesis, commonly results in unwanted side products associated with incomplete peptide chains. Here, LC–electrospray MS has been used to examine a crude of synthesis of carbetocin, a peptide with recognized therapeutical application for stimulation of uterine contractions to facilitate parturition. The molecular masses of various side products obtained within the crude of synthesis have been determined and these side products have been identified on the basis of their molecular masses. The knowledge of the impurities present in the crude peptide permits the optimization of the synthetic procedure.

Keywords: Pharmaceutical analysis; Carbetocin; Peptides

1. Introduction

Synthetic peptides are becoming increasingly important as drugs in the pharmaceutical industry. Carbetocin [CAS name: 1-butanoic acid-2-(*O*-methyl-L-tyrosine)-1-carbaoxytocin] is a synthetic structural analogue to oxytocin used in human and veterinary medicine for stimulation of uterine contractions to facilitate parturition and for promotion of milk let-down, as lactation agent. Carbetocin is used instead of oxytocin because it has a longer duration of action.

Advances in biotechnology have provided the ability to prepare peptides for therapeutic purposes. However, stepwise solid-phase peptide synthesis (SPPS), the widely used method of synthesis first introduced by Merrifield [1] commonly results in unwanted side products associated with incomplete peptide chains, and their use has called for efficient isolation of peptides from various impurities [2–6]. Such incomplete or, as they are commonly called, difficult sequences are provoked by problems of swelling and aggregation of the growing peptide chains in the course of synthesis.

Analytical and preparative liquid chromatography (LC) together with capillary electrophoresis (CE), which are commonly used to purify and characterize new synthesis products [2,4,7,8], are not always sufficient to obtain unambiguous analytical infor-

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mation regarding all the components of the reaction mixture.

Fast, reliable identification of side-products associated with the target peptide is desirable in order to design and improve the synthesis procedures of peptides and also is needed for the commercialization of the peptide product. LC coupled to mass spectrometry (MS) using an electrospray ionization interface (LC–ESI-MS) provides a sensitive method for the analysis of peptides in complex matrixes [9–12]. MS, based on precise measurement of the mass-to-charge ratio, is now considered to be a significant aid in peptide and protein structure determination [13–17]. In the past few years, new ionization technologies have been developed, electrospray ionization being one of the most popular ionization techniques because of its sensitivity and its ability to analyze large, thermally labile biomolecules and to provide accurate molecular mass measurements using mass analyzers with limited range such as quadrupoles ($m/z<2500$) [18–20].

Here, liquid chromatography coupled to electrospray mass spectrometry has been used to examine a crude of synthesis of carbetocin, obtained by solid-phase methodology. The molecular masses of various side products obtained within the crude of synthesis have been determined and these side products have been identified on the basis of their molecular masses. The knowledge of the identity of these impurities present in the crude peptide may be helpful to avoid the synthetic routes responsible for such undesirable products and, so, to minimize their formation which will also make easier to obtain the purified therapeutic product.

2. Experimental

2.1. Reagents

Water with a conductivity lower than 0.05 μS cm^{-1} and acetonitrile (Merck, Darmstadt, Germany) were of HPLC grade. Trifluoroacetic acid (TFA), potassium bromide and potassium hydrogenphthalate (dried at 110°C before use) were all analytical reagent grade from Merck.

Crudes of synthesis of carbetocin were supplied by Lipotec (Barcelona, Spain). Carbetocin crudes were dissolved in an acetonitrile–water (25:75) mixture, containing 0.1% (v/v) TFA, at concentrations of approximately 2 mg/ml and 3 mg/ml.

2.2. Apparatus

For the LC–UV experiments an ISCO (Lincoln, NE, USA) Model 2350 chromatographic pump with a Valco injection valve with a 10-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 (BC. Aplicaciones Analíticas, Barcelona, Spain) column was used at room temperature.

The emf values used to evaluate the pH of the mobile phase [21] were measured with a Model 2002 potentiometer (Crison Instruments, Barcelona, Spain) and a 8102 ROSS combination electrode (Orion Research, Boston, MA, USA) with a precision of ±0.1 mV. The potentiometric system was calibrated using a standard reference solution of 0.05 mol/kg potassium hydrogenphthalate [22], whose reference pH values are known in the acetonitrile–water mixtures studied [23].

For the LC–ESI-MS experiments a VG Platform II quadrupole mass spectrometer from Micromass (Manchester, UK) equipped with a nebulizer assisted electrospray source was used. The high-flow nebulizer was operated in a standard mode with N_2 as both nebulizing (15–20 l/h) and drying (400 l/h) gases. Instrument control and data analysis were accomplished using MassLynx application software from Micromass. The mass range was m/z 300–1200. All spectra were obtained in the positive ion mode. Generally, the mass data represent the average of three separate measurements.

Two Phoenix 20 HPLC pumps (CE Instruments, Milan, Italy) with a Rheodyne 7125 injection valve (Cotati, CA, USA) and a 20-μl sample loop were connected to the MS system.

Separation was performed on a Kromasil C_8 column with 1 ml/min flow-rate, split to allow 20 μl/min effective flow into the source.

2.3. Optimization of the source and analyzer parameters

The optimization of the source and analyzer parameters [24] was performed using electrospray ionization of 50 μg/ml crude solution in acetonitrile–water (25:75), 0.1% (v/v) TFA, introduced directly into the ESI source, at a flow-rate of 20 μl/min. Parameters were optimized in order to obtain the best signal stability and the highest sensitivity of the target peptide carbetocin. Optimum conditions were as follows: capillary voltage, 4250 V; counter electrode voltage, 500 V; sample cone voltage, 60 V; source temperature, 60°C; ion energy, 3.5 V. Fragmentation was also investigated under these working conditions. For some impurities, significant fragmentation was observed at a sample cone voltage of 60 V. It can be observed that at lower voltages ESI yields simple mass spectra with negligible fragmentation allowing correct identification of the molecular identities, which is essential for the characterization of impurities. For this reason, spectra were obtained using extraction voltages of 30 V. In order to partially counteract the decrease of sensitivity, a higher source temperature (70°C) and ion energy (5 V) were used.

2.4. Procedure

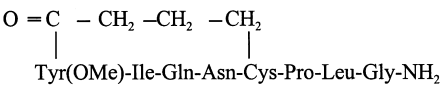
For the identification of the different side products of the crude of synthesis, 2 mg/ml and 3 mg/ml solutions of carbetocin crude were injected into the LC–ESI-MS system, using acetonitrile–water (25:75) containing 0.1% TFA as mobile phase. The pH measured in this mixture was 1.9 ESI spectra of carbetocin and associated side products were obtained at the optimum conditions and at full scan mode.

3. Results and discussion

The synthetic crude obtained was first examined by analytical LC. The resulting UV and total ion current (TIC) chromatograms are shown in Figs. 1 and 2, respectively. They contain a major peak associated with the target peptide carbetocin as well as a number of peaks corresponding to unidentified peptidic substances.

ESI spectra associated with all the chromatographic peaks observed in the TIC are showed in Fig. 3. The mass-to-charge ratios observed, the respective charged states and the monoisotopic molecular masses measured for each substance are summarized in Table 1, as well as the assignment of each ion on each peptide. The molecular masses were calculated from both the singly and doubly charged states present in spectra [25] by means of the algorithm supplied by the manufacturer.

In order to identify the target peptide and most associated side products, it is important to know the method by which the peptide was synthesized and the reagents added in the synthesis process to protect the different functional groups. In SPPS, protection of the α-amino group of the amino acid that has to be coupled is mandatory in order to avoid consecutive additions [26]. Similarly, the amino or other nucleophilic groups in the side chains, must be blocked to avoid their reactivity. In our case, carbetocin was prepared by solid-phase peptide synthesis using the following protection scheme: 9-fluorenylmethoxycarbonyl group (Fmoc) to protect the α-amino group and triphenylmethyl group (Trt) to protect the sulfhydril group of cysteine. After completion of the sequence with the introduction of $Cl–CH_2–CH_2–CH_2–COOH$, the peptide was cleaved from the solid support with simultaneous deprotection of the side chains using TFA and then was cyclated using DIEA (*N*-ethyldiisopropylamine) to obtain the final product



Carbetocin

The spectrum of the target peptide, $t_R = 50.6$, is shown in Fig. 3a. The following charge state distribution can be seen: the m/z 988.2 corresponding to the $[M+H]^+$ charge state and the m/z 494.8 corresponding to the $[M+2H]^{2+}$ charge state. These ions are associated with the molecular mass 987.2 which is in good agreement with the calculated monoisotopic molecular mass of the carbetocin, 987.5. The ion of m/z 485.4 is attributed to a fragmentation of the carbetocin, Asn–Cys–Pro–

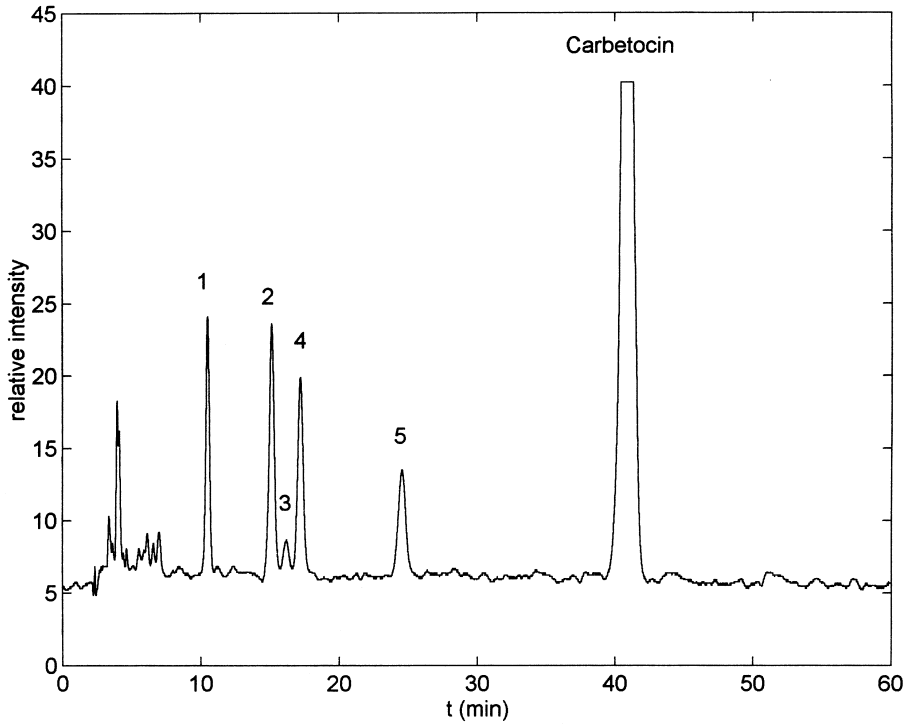


Fig. 1. UV chromatogram of the carbetocin crude of synthesis. Mobile phase: acetonitrile–water (25:75), 0.1% (v/v) TFA. Amount injected: 20 μg of the target peptide. Peaks 1–5 are impurities present in the crude.

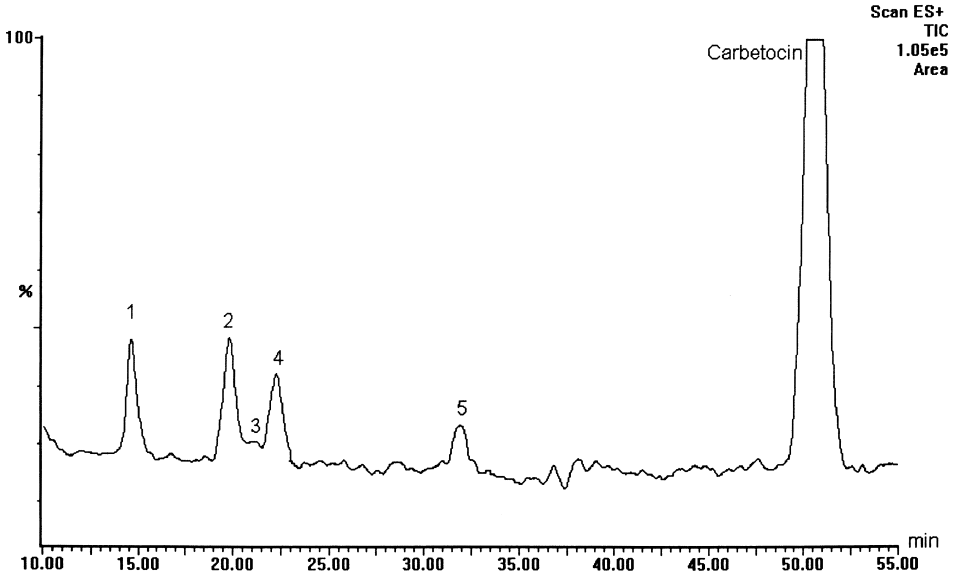


Fig. 2. Total ion current (TIC) chromatogram of the carbetocin crude of synthesis. Mobile phase: acetonitrile–water (25:75), 0.1% (v/v) TFA. Amount injected: 60 μg of the target peptide. Retention times of the impurities: impurity 1, $t_R = 14.6$ min; impurity 2, $t_R = 19.8$ min; impurity 3, $t_R = 21.0$ min; impurity 4, $t_R = 22.3$ min; impurity 5, $t_R = 32.2$ min; carbetocin, $t_R = 50.6$ min.

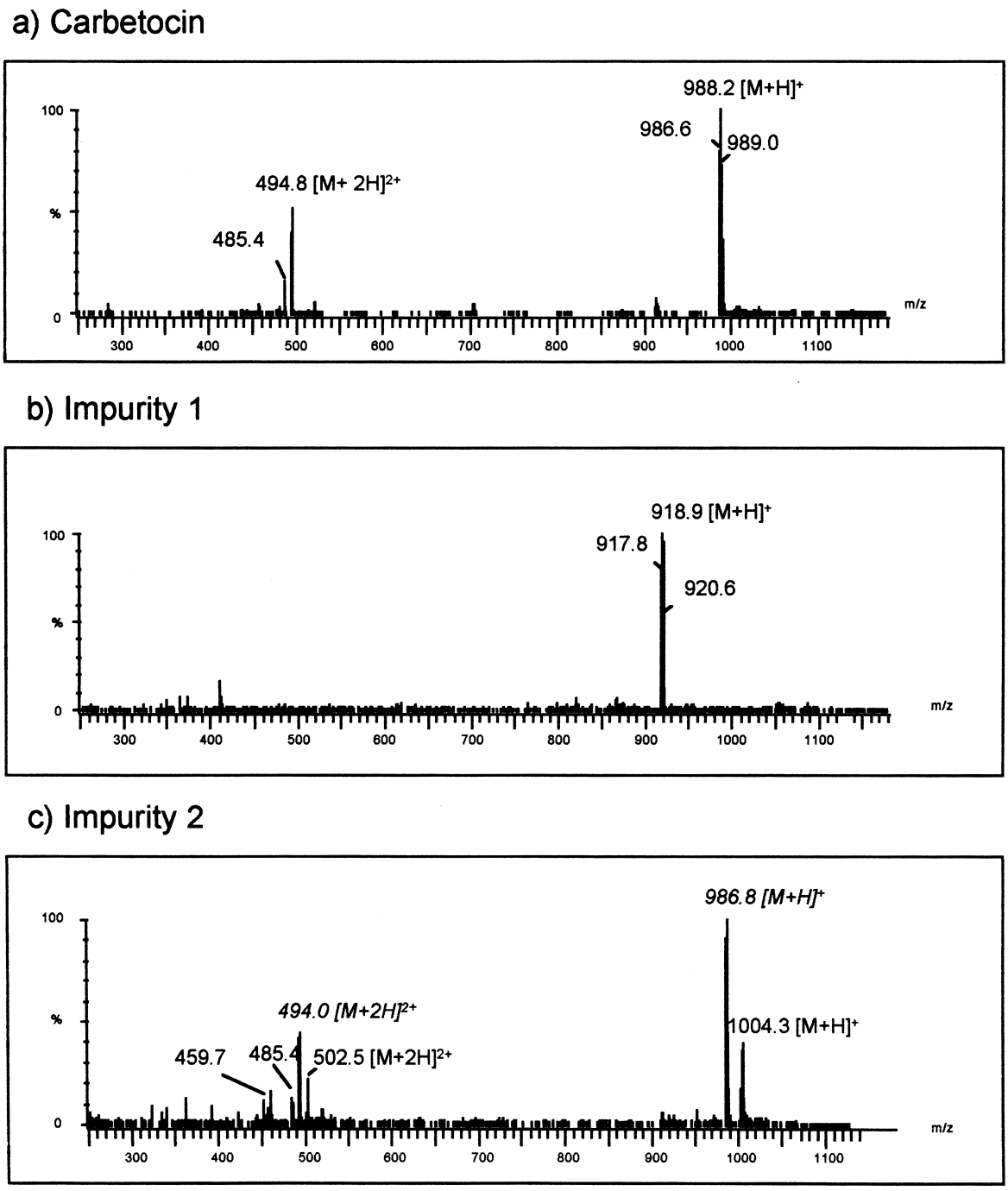
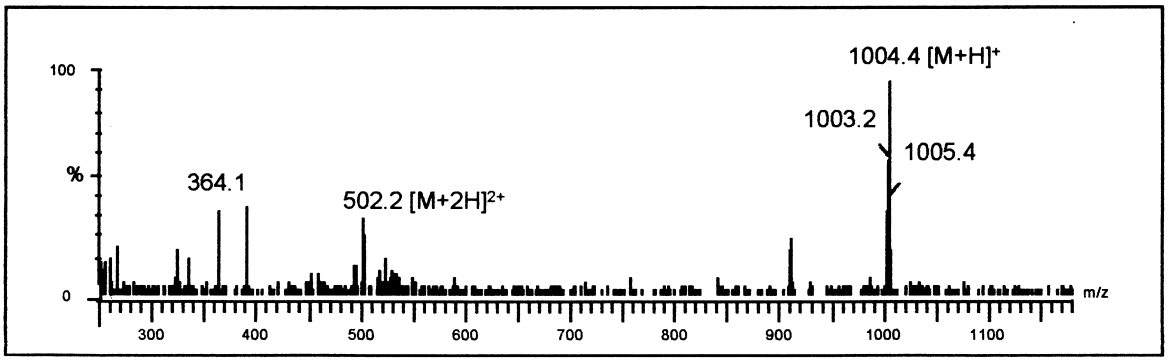
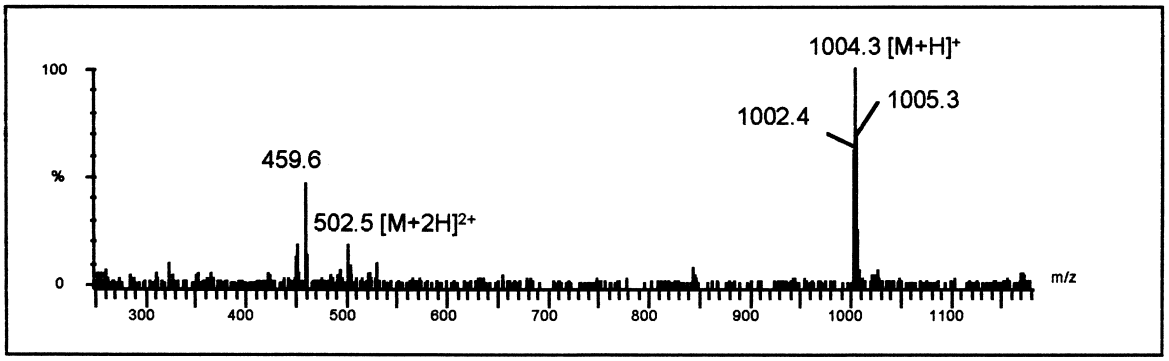


Fig. 3. Electrospray mass spectra of (a) target peptide carbetocin, (b) impurity 1, (c) impurity 2, (d) impurity 3, (e) impurity 4, (f) impurity 5.

d) Impurity 3



e) Impurity 4



f) Impurity 5

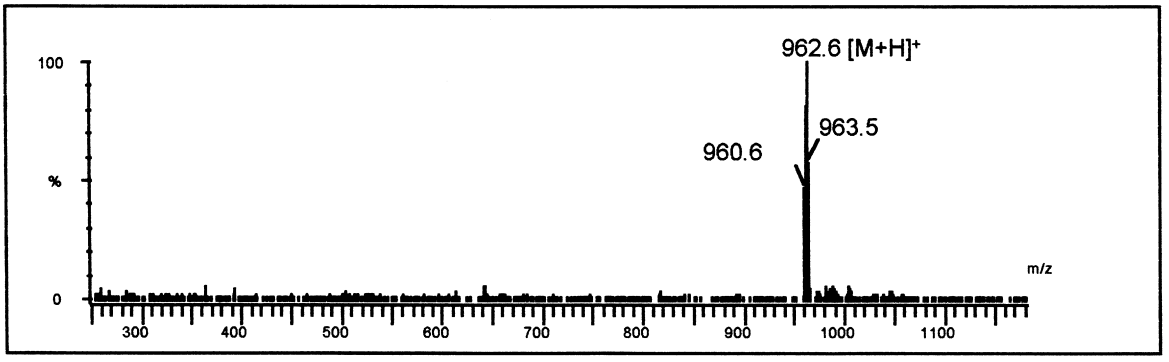
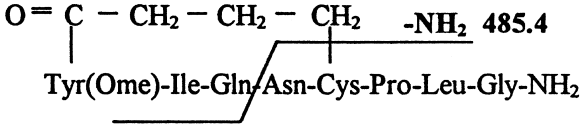


Fig. 3. (*continued*)

Table 1
Measured relative molecular masses (M_r) and proposed sequences associated with the TIC peaks in Fig. 2

Impurity	t_R (min)	Observed m/z	Measured M_r	Proposed sequence
1	14.6	918.9	917.9	Tyr(OCH_3)–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2
2	19.8	986.8 494.0	985.9	Carbetocin, racemic
		1004.3 502.5	1003.0	Sulfoxide of carbetocin
3	21.0	1004.4 502.2	1002.9	Sulfoxid of carbetocin, racemic
4	22.3	1004.3 502.5	1003.0	Sulfoxid of carbetocin, racemic
5	32.2	962.6	961.6	Fmoc–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2
Carbetocin	50.6	988.2 494.8	987.2	

Leu–Gly–NH_2, which has lost the α-NH_2 group of the asparagine.



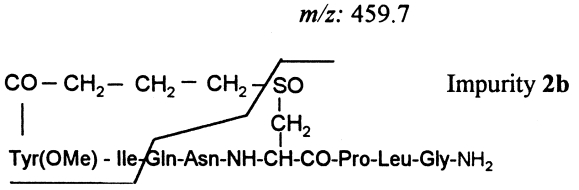
The identification of the side products is solely based on the observed mass differences between the mass of the carbetocin, 987.5, and the mass of the side product.

Fig. 3b shows the spectrum associated with the chromatographic peak 1 (t_R=14.6 min) which contains only one charge state $[M+H]^+$ yielding the relative molecular mass of 917.9. This mass is attributed to the incomplete coupling of Cl–CH_2–CH_2–CH_2–COOH, which leads to the next by-product:

Tyr(OCH_3) − Ile − Gln − Asn − Cys − Pro − Leu − Gly − NH_2 Impurity 1

The calculated mass of this sequence, 917.4, is in good agreement with the measured one.

The spectrum of Fig. 3c shows a bigger complexity, due to the coelution of two substances at the same t_R=19.8 min (peak 2). The first (in italic letter in Fig. 3c), presents two dominant charge states m/z 986.8 $[M+H]^+$ and m/z 494.0 $[M+2H]^{2+}$, which give a value of M_r=985.9 (difference of mass compared with carbetocin, ΔM=1.6 u). This mass could be attributed either to a product of reduction of the carbetocin or, taking into account the presence in the spectrum of the fragment m/z 485 associated to a fragmentation of carbetocin (see previous structure), to a diastereoisomer of the target peptide. The second co-eluting substance also shows two charge states, m/z 1004.3 $[M+H]^+$ and m/z 502.5 $[M+2H]^{2+}$, yielding the molecular mass M_r=1003.0, attributed to the oxidation of the sulfhydryl group of cysteine residue, because the difference of mass compared with carbetocin is of 15.5 u. In the spectrum, the mass-to-charge ratio m/z 459.7 can also seen. This corresponds to a fragment of the earlier sulfoxide, as shown in the following scheme:



The spectra associated with chromatographic peaks 3 (t_R=21.0) and 4 (t_R=22.3 min) show

similar profiles as can be seen in Fig. 3d and e: the charge-to-mass ratios $m/z=1004.3$ $[M+H]^+$, and $m/z=502.5$ $[M+2H]^{2+}$, associated with the same molecular mass $M_r=1003.0$, as well as the characteristic fragmentation m/z: 459.7 can be observed. These spectra are essentially the same as that of impurity 2b, but at different retention times. Therefore, impurities 3 and 4 have been attributed to diastereoisomers of the sulfoxide of carbetocin.

In fact, the chromatogram of ion m/z: 1004.3, Fig. 4, shows the presence of this mass at different retention times, which suggests that racemization of this molecule is considerable. Among the undesired reactions that accompany the different operations of peptide synthesis, racemization is the most general cause for concern [27].

In the SPPS it is also frequent to obtain as impurities partially protected products [26,28]. In the deprotection steps, a portion of blocked peptide chains is left fully protected after the exposure to the deblocking reagent because the accessibility of some functional groups is not always perfect. This is the case of impurity 5 (peak with $t_R=32.2$ min), which shows a mass spectra with a singly protonated species corresponding to a measured mass of $M_r=$ 961.6, Fig. 3f. This molecular mass is tentatively attributed to the following sequence:

Fmoc − Ile − Gln − Asn − Cys − Pro − Leu − Gly − NH_2 Impurity 5

with a calculated molecular mass of 962.0 which is in good agreement with the measured one.

Imperfections in the removal of blocking groups lead to the formation of chains from which one of the amino acid residues is absent. Such materials were designated ''failure sequences'' or ''deletion sequences'' [28]. The presence of deficient sequences in the synthetic material creates serious problems in purification, because the properties of such impurities are generally quite similar to those of the target compound [29].

The data presented here prove the capability of the LC–ESI-MS system to furnish fast and reliable information on the various products of the solid-phase synthesis of peptides. Such information allows fast identification of the target peptide and most associated side products.

The more common impurities present in the crude of synthesis of carbetocin can be attributed to: oxidized species due to the presence of a cystine residue, racemization – an inherent problem in liquid and solid-phase synthesis – and failure or deletion sequences due to incomplete deprotection.

The knowledge of the identity of the impurities

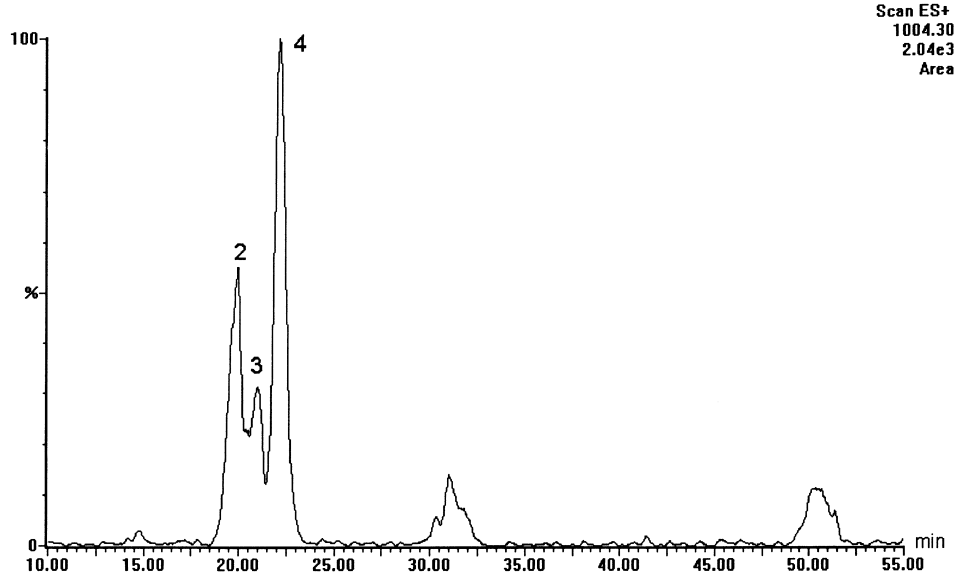


Fig. 4. Single ion monitoring (SIM) chromatogram of $m/z=1004.3$.

present in the crude of synthesis allows the chemist to design and improve the synthesis procedures of peptides and to obtain the desired pure therapeutic product.

Acknowledgements

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