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Original Article

The application of peptide sequencing for characterization of cetrorelix and its related impurities

Shirin Hooshfar [1, 2*], Seyed Alireza Mortazavi [2], Samaneh Ghassemi[2], Sara Ghassemi[2], Farzad Kobarfard [3, 4]

[1]Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602-2352, USA.
[2]Department of Pharmaceutics, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[3]Department of Medicinal Chemistry, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[4]Central Research Laboratories, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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ABSTRACT

Electrospray ionization mass spectrometry (ESI-MS) was used to evaluate the structure of cetrorelix and the related impurities generated throughout the solid phase peptide synthesis (SPPS) reactions by Fmoc strategy. It was concluded that these by-products are formed as a result of incomplete removal of the protecting groups placed on side chains during synthesis. The impurity with 56 Da mass difference from cetrorelix was determined to be the result of failure in deprotection of L-serine residue and the impurity at 252 Da higher m/z than cetrorelix was due to the incomplete removal of Pbf from L-arginine. Peptide sequencing using ESI-MS allowed for rapid and reliable identification and characterization of the target peptide and its impurities. Furthermore, the information obtained from this method can offer a significant advantage for purity assessment of synthetic peptides which are used as therapeutic or research tools and it can also contribute a significant amount toward designing more efficient synthesis pathways for these molecules.

Keywords: cetrorelix, synthetic impurities, peptide sequencing, solid phase peptide synthesis

INTRODUCTION

Cetrorelix (N-acetyl-3-(2-naphthalenyl)-D-alanyl-4-chloro-D-phenylalanyl-3-(3-pyridinyl)-D-alanyl-L-seryl-L-tyrosyl-N5-(aminocarbonyl)-D-ornithyl-L-leucyl-L-arginyl-L-prolyl-D-alaninamide) is a synthetic decapeptide with gonadotropin-releasing hormone (GnRH) antagonistic activity, and it is currently one of the main therapeutics for the treatment of infertility. It may also be prescribed to patients with benign prostatic hypertrophy or sexual hormone-dependent tumors [1-6].

In the past several years, solid phase peptide synthesis (SPPS) has become a major technique for production of therapeutic peptides [7]. Various side-reactions can occur during SPPS, resulting in several types of impurities in the final peptide product. These undesired reactions may include: oxidation and reduction of amino acids, amino acid deletions, amino acid insertions, side chain reactions and amino acid modifications during cleavage [8, 9]. Such impurities are often present even after purification, and they have to be carefully characterized and quantitated to meet the pharmaceutical regulatory requirements [8, 10, 11]. Furthermore, knowledge of the structure of related impurities could be helpful for optimizing the synthesis method, including coupling and cleavage steps [12].

Electrospray ionization mass spectrometry (ESI-MS) is a very powerful technique for interrogating the structure and sequence of synthetic peptides. The most interesting application of ESI-MS in this field is peptide sequencing [13, 14]. In this study, an ESI-MS method for sequencing of cetrorelix was developed and used for characterization of the target peptide and its related impurities. To the best of our knowledge, cetrorelix does not have an official monograph in any pharmacopoeia and no report of characterizing the related synthetic

***Corresponding Author Address:** Dr. Shirin Hooshfar, University of Georgia, Collage of Pharmacy, Department of Pharmaceutical and Biomedical Sciences, West Green St., Athens, GA., 30602; E-mail: hooshfar@uga.edu*

impurities of cetrorelix has ever been published. Additionally, cetrorelix is a synthetic peptide with five unnatural amino acids in its sequence [2]. Currently, most researchers use database searching to infer the peptide sequence from its fragmentation spectra. However, for synthetic peptides with unnatural amino acids in their structures this approach is bound to fail [13, 15].

The present study was aimed at the development of a peptide sequencing method using ESI-MS/MS for the identification of cetrorelix and its related peptide impurities formed during SPPS. Generally, the structure of related impurities can be found in the official monographs for most of small molecule drugs [16-19]. However, the structure of impurities for many synthetic peptide drugs are totally unknown and some of them do not even have an official monograph. Therefore, the findings of this research can provide a general framework for investigation of the structure and characterization of the sources that may introduce impurities to the final SPPS product. Considering the ever-increasing number of synthetic peptides used as therapeutics or probes for the elucidation of basic cellular pathways, this understanding will have important implications.

MATERIALS AND METHODS

Chemicals and reagents: Fmoc amino acids with standard side chain protecting groups, Rink amide 4-methylbenzhydrylamine resin (Rink Amide MBHA) and HATU were obtained from GL Biochem (Shanghai, China). Solvents and other reagents for peptide synthesis were of LC-MS or analytical grade and purchased from Merck (Hohenbrunn, Germany).

Peptide synthesis and purification: Cetrorelix was synthesized on a Rink Amide MBHA solid phase support which allowed for the cleavage of peptide amide in acid conditions with the concomitant deprotection of the side chains. The elongation of the peptide chain was carried out using the Fmoc/tBu strategy with HATU as the coupling reagent. The Fmoc groups were removed via treatment with 20% piperidine in dimethylformamide (3 times), and the resulting peptidyl resin was treated at room temperature with a mixture of trifluoroacetic acid / triisopropylsilane /H2O (95/2.5/2.5, v/v/v, 10 ml/1g resin) for 140 minutes. Peptides were precipitated by filtration into cold methyl tert-butyl ether and vacuum-dried. The crude product was dissolved in 30% acetic acid and freeze-dried. The final product was purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) on a Eurospher C18 column (250×16 mm, 10 µm), using a gradient (15–40%) of acetonitrile/water containing 0.1% TFA over a period of 75 min at a flow rate of 15 ml/min.

The peak detection was performed in UV light at 270 nm, followed by a manual fractionation of cetrorelix and its two major impurities. Acetonitrile was removed from the fractions on a rotary evaporator at 50ºC under a water jet vacuum. The remaining peptides were lyophilized and subsequently characterized by mass spectrometry.

Peptide Sequencing: Individual solutions of cetrorelix and its two related impurities were prepared by dissolving 1.0 mg of each compound in 100 mL of methanol to obtain a concentrations of 10 µg/ml. These solutions were then infused to the ESI source of an Agilent Triple Quad mass spectrometer 6410 (m/z range: 20–2000) using the Agilent 1200 Binary SL liquid chromatography system (Waldbronn, Germany). Flow injection analysis was performed by an injection volume of 10 µl at a flow rate of 0.3 ml/min of a mixture of methanol/water (90/10, v/v).

For the collision induced dissociation (CID) experiments, the collision energy of 25 eV was used for the doubly charged ions of cetrorelix and impurity A and the collision energy of 40 eV was applied to the doubly charged ion of impurity B. The ESI product ion scan was performed over the m/z range of 50–2000. MS-source parameters were as follows: Capillary and fragmentation voltages were adjusted to 4000 and 135 volts, respectively. Nitrogen was used as drying and nebulizing gas at 350ºC temperature and 50 psi pressure.

RESULTS AND DISCUSSION

The MS spectra of three major fractions collected from the HPLC separation of the SPPS product (Fig. 1) were obtained at MS-scan mode from 50 to 2000 m/z. Fig. 2 shows the MS spectra of cetrorelix, impurity A and impurity B. The mass values of the impurities A and B were 56 and 252 Da higher than the mass of cetrorelix, respectively. 56 Da mass difference between impurity A and cetrorelix could be indicative of incomplete side-chain deprotection reaction of serine or tyrosine resulting in a peptide with a tBu (tert-butyl) protecting group still on its side chain. The 252 Da mass increase for impurity B is pointing towards a peptide containing an uncleaved Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). This suggests that the side chain protecting group (Pbf) of arginine has not been completely removed during the cleavage process. Fig. 3 demonstrates the product ion spectrum of cetrorelix with the molecular mass of 1431 Da for the protonated

peptide, obtained by fragmenting a doubly charged ion at m/z of 716. A summary of all fragments which were found in the product ion spectrum, as well as the structure of cetrorelix is presented in Fig. 4.

The fragmentation pattern of peptides depends on the collision energy, the number of charges being carried by the peptide and the peptide sequence. Generally, peptides are fragmented at the amide bonds and then produce b- and y-ion series. A summary of theoretical fragment ions of cetrorelix is presented in Table 1. According to the results summarized in Fig. 4, some product ions of b- and y-ion series were not observed. It reflects the simple fact that subtle differences in the amide bonds caused by the different nature of amino acid side chains and their positions in the peptide sequence, generates both favorable and unfavorable fragmentation sites. Because the population of protonated peptide ions is finite, favorable fragmentation at some amide bond must be compensated by a reduction in fragmentation at other unfavorable sites [13]. Nevertheless, sufficient fragmentation information was provided by the obtained spectra to unambiguously confirm the sequence of cetrorelix.

Fig. 5 and 6 show the product ion spectra of impurities A and B, obtained via fragmentation of doubly charged ions at m/z 744 (impurity A) or 842 (impurity B). A summary of all fragments observed in the product ion spectra of these impurities and their hypothetical structures are demonstrated in Fig. 7 and 8.

The product ion spectra of impurity A contained ions of both the b and y series which confirmed the expected sequence of the peptide. The fragment ion b_4 was observed at m/z = 714 that indicated the presence of tBu group on the serine residue. In addition, the m/z values for y_5 and y_6 fragment ions were unaffected ruling out the possibility of tBu attachment to the tyrosine.

Side chain protecting groups of amino acids are usually hydrophobic molecules which are easily cleaved from the side chain in the collision cell during CID. Therefore, the product ion spectra of peptides with protecting groups often contains fragment ions derived from the protecting group. Fig. 6 shows the product ion spectrum of impurity B. A fragment ion was observed at m/z of 252.8 which indicated collision induced cleavage of Pbf from the parent ion. Furthermore, y_3, z_3, x_3 and y_6 fragment ions of impurity B were all 252 Da heavier than the analogous fragment ions of cetrorelix. This observation clearly confirmed the incomplete removal of Pbf from the arginine residue.

Synthetic peptides are not easily classified into either one of “small molecules” or “biologics” categories, because of their varying size and amino acid sequences. Therefore, many regulatory challenges still remain to be addressed for peptides especially with respect to their impurities and characterization requirements. Peptide sequencing provides exhaustive information about amino acid composition that can be considered as an efficient alternative to traditional amino acid analysis methods. The very small amount of peptide required to conduct the experiment, is a major advantage of this method. As explained above, we were able to characterize two impurities of cetrorelix which formed during the process of solid phase peptide synthesis. The method was easily implemented using impurities at microgram scale and it was proven to be reproducible enough to be considered as an official compendial method for characterization of cetrorelix and its impurities.

CONCLUSION

The product-related impurities of cetrorelix were characterized by a CID mass spectrometry method as peptides containing uncleaved side chain protecting groups. Separation and identification of these side products may be regarded as a key tool for the optimization of the synthetic procedures, as well as fulfillment of the necessary regulatory requirements for cetrorelix as a peptide drug API. Furthermore, this research presented a simple and fast method for the determination of the most common impurities that can occur during any SPPS procedure, i.e., products of incomplete side chain deprotection. Briefly, the impurities can be very quickly analyzed by CID-MS/MS and the presence of side chain protecting groups (such as Pbf) in their product ion spectra will indicate the incomplete removal of those protecting groups.

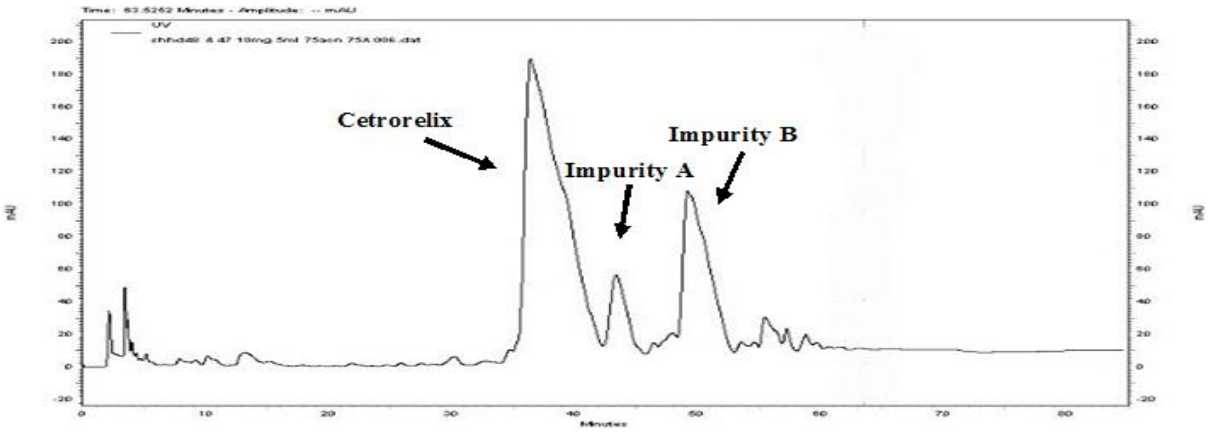

Fig. 1. Preparative chromatogram of the crude peptide product at 270 nm.

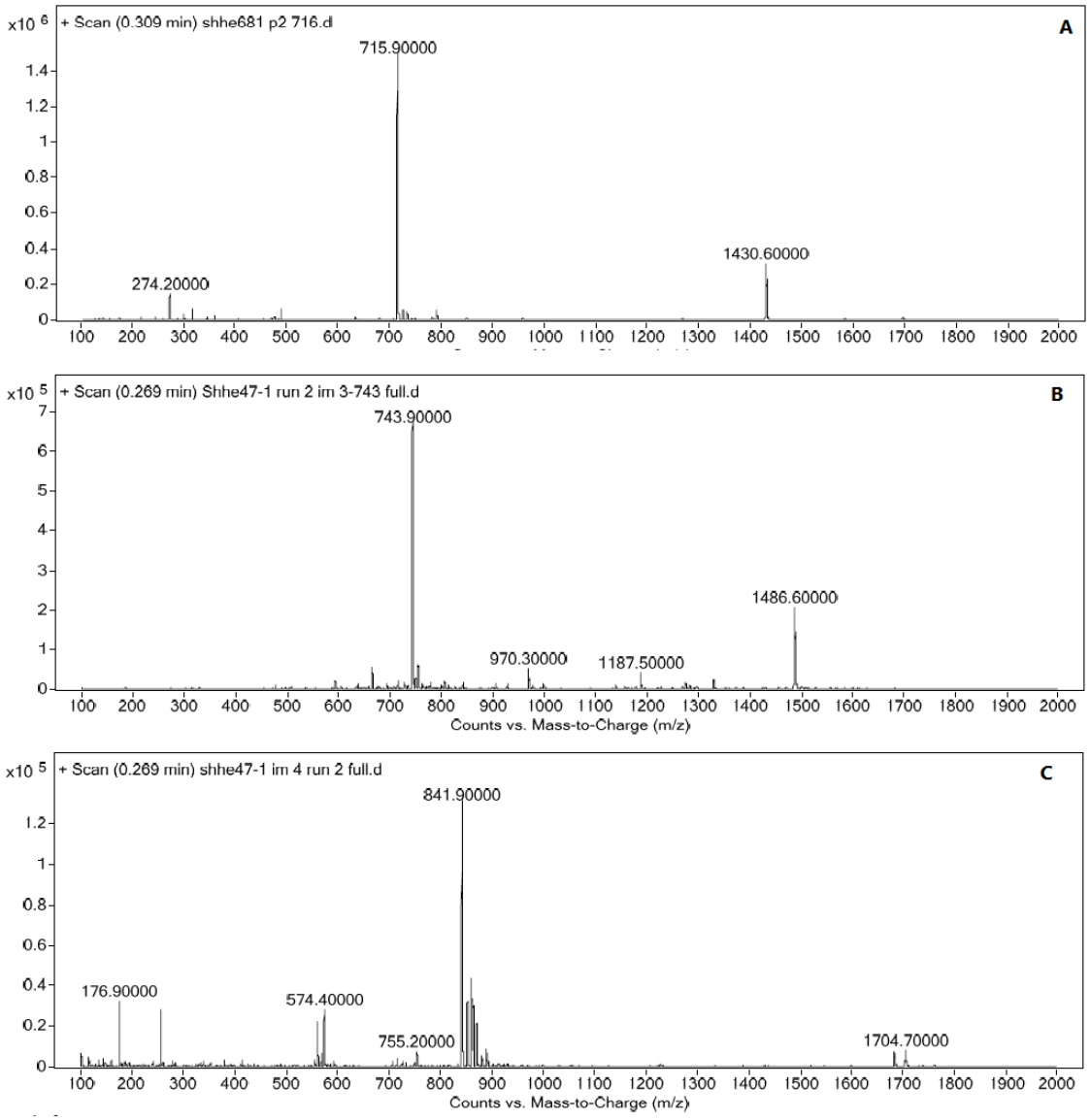

Fig. 2. Mass spectra of cetrorelix and its synthetic impurities. A: The MS spectrum of cetrorelix (mw: 1431.06 g/mol) in full scan mode ($[M+H]^+$ at m/z 1430.6 and $[M+2H]^{++}$ at m/z 715.9). B: The MS spectrum of impurity A in full scan mode ($[M+H]^+$ at m/z 1486.6 and $[M+2H]^{++}$ at m/z 743.9). C: The MS spectrum of impurity B in full scan mode ($[M+Na]^+$ at m/z 1704.7 and $[M+2H]^{++}$ at m/z 841.9).

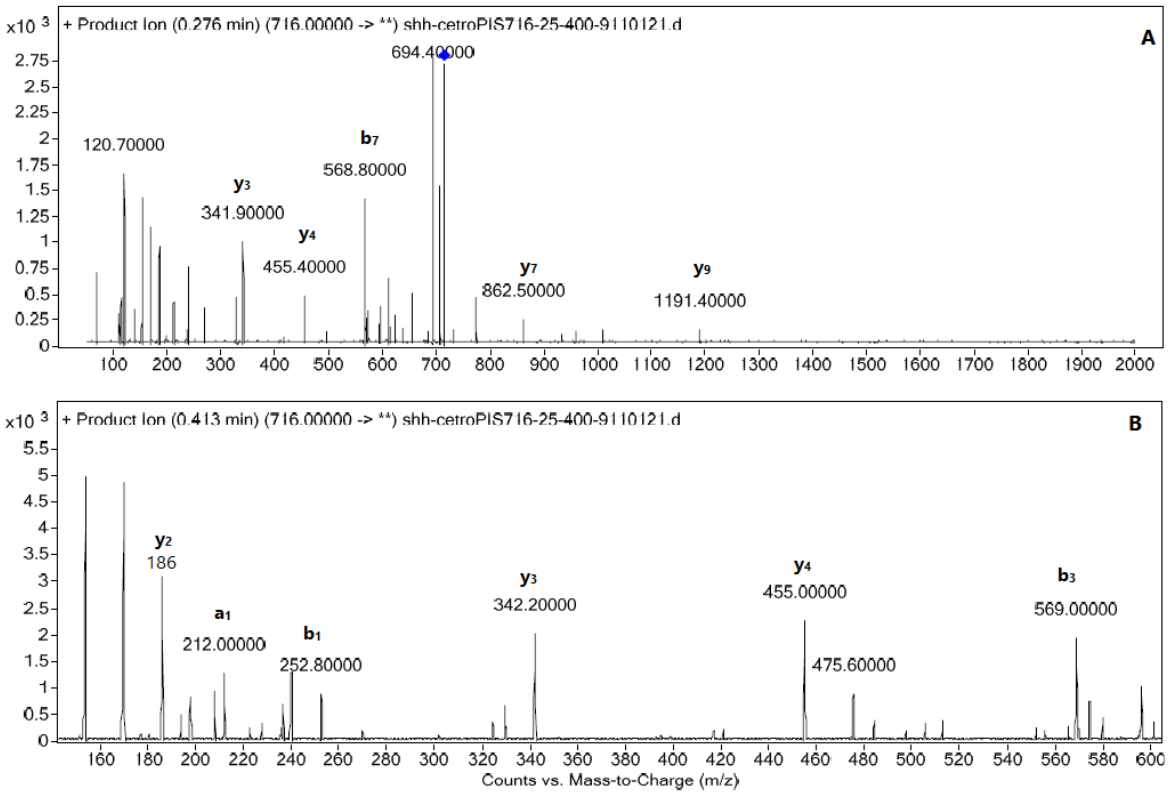

Fig. 3. A: The product ion spectrum of cetrorelix. The m/z of the precursor ion was 716 (CID: 25). B: The portion of spectrum from 100 to 600 m/z is further zoomed.

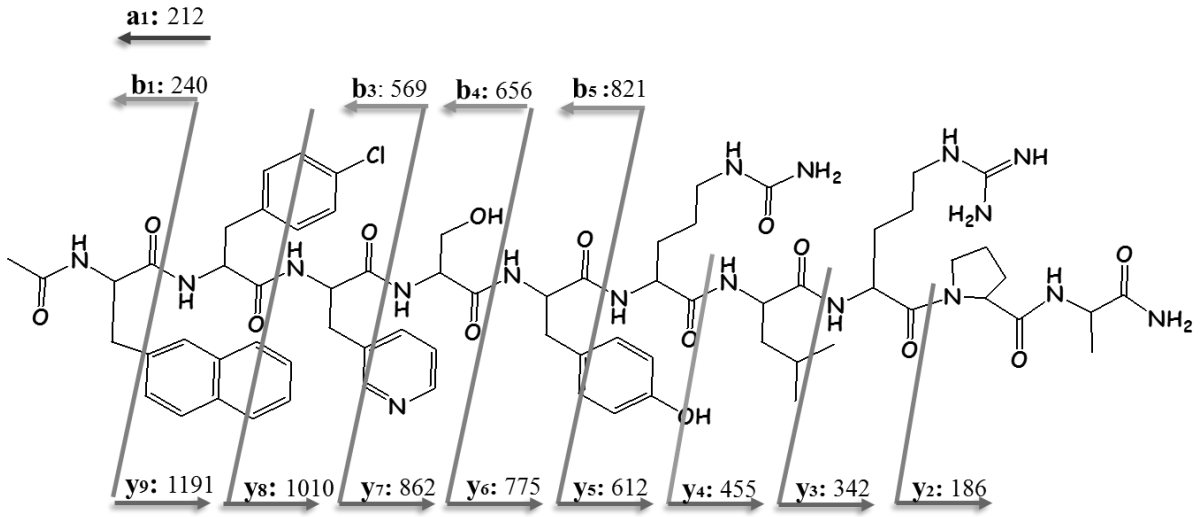

Fig. 4. Structure of cetrorelix and all fragment ions that were found in its product ion spectrum.

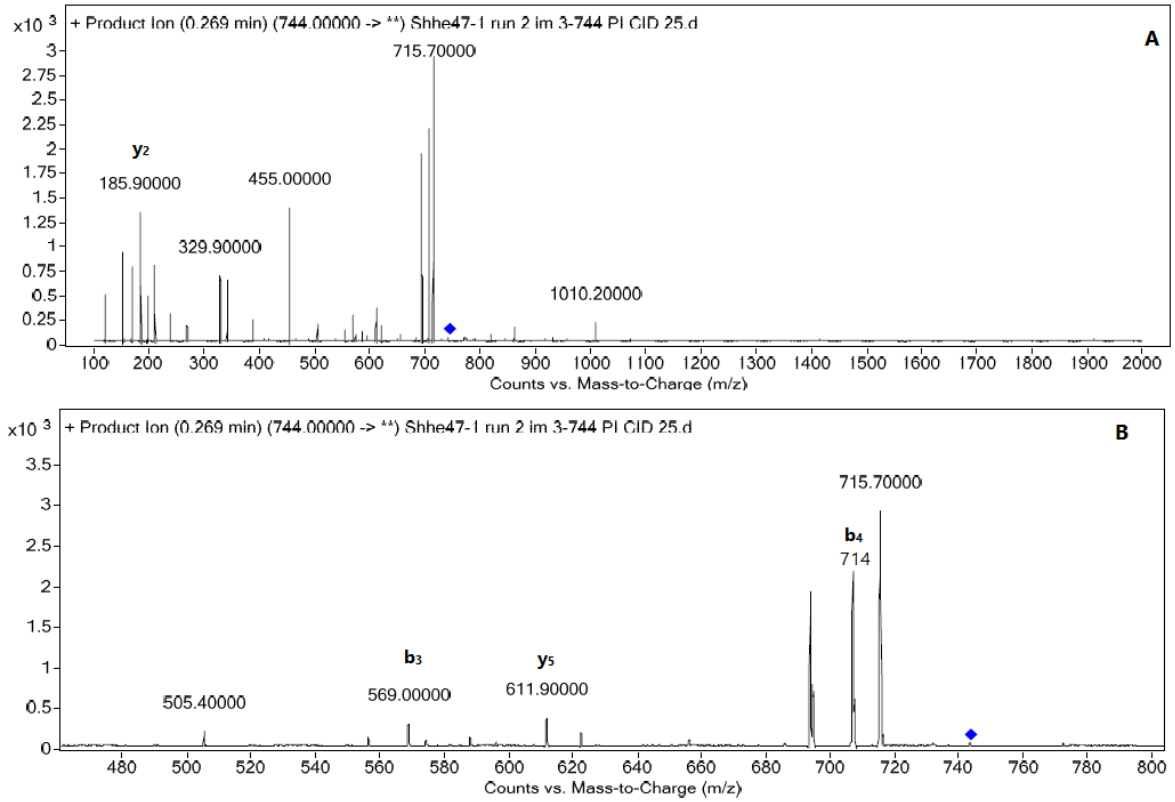

Fig. 5. A: The product ion spectrum of impurity A. The m/z of the precursor ion was 744 (CID: 25 eV). B: The portion of product ion spectrum of impurity A is further zoomed into from m/z 450 to 760.

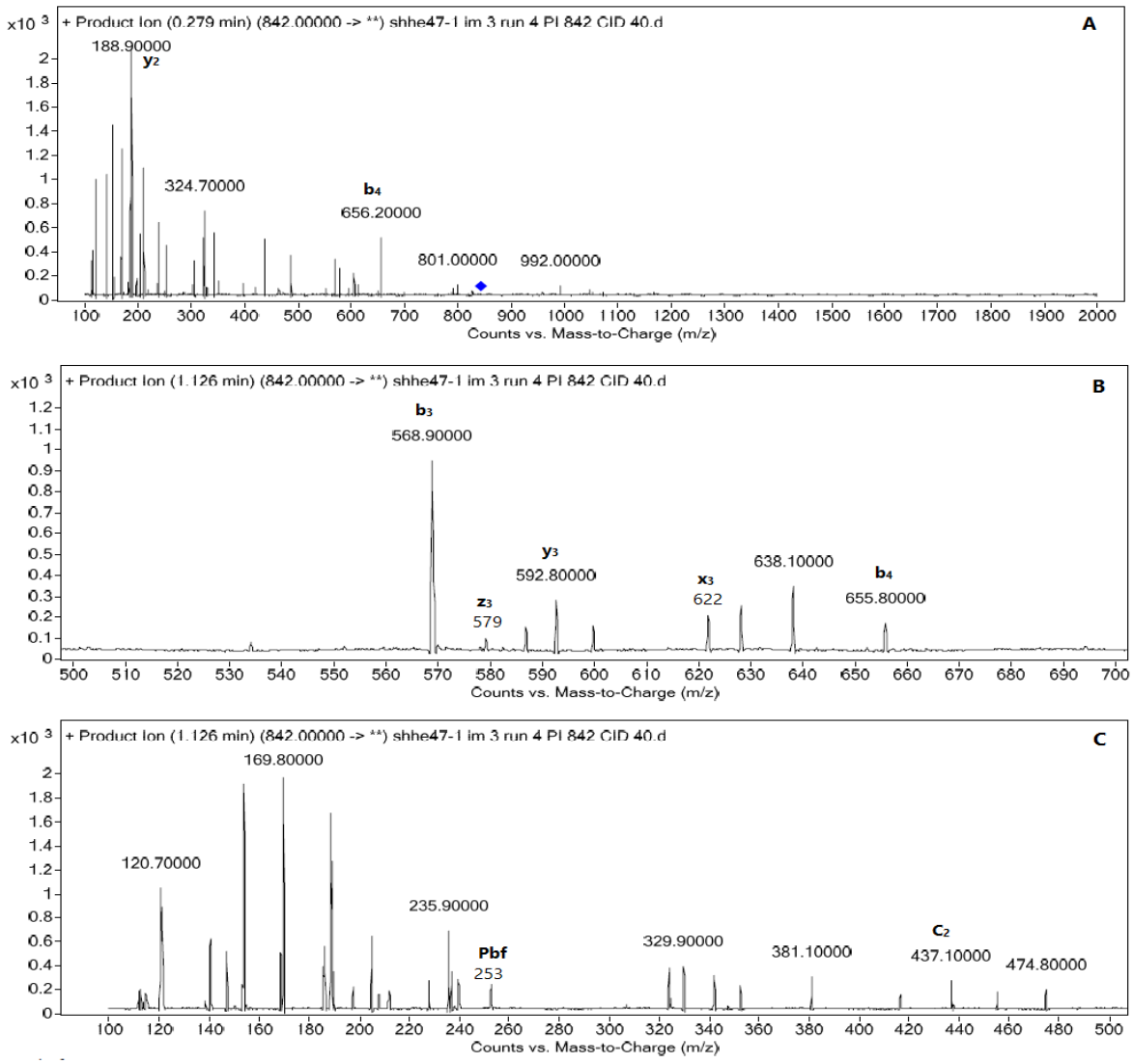

Fig. 6. A: The product ion spectrum of impurity B. The m/z of the precursor ion was 842 (CID: 40 eV). B: Zoomed product ion spectrum of impurity B from 520 to 700 m/z. C: Zoomed product ion spectrum of impurity B from 220 to 480 m/z.

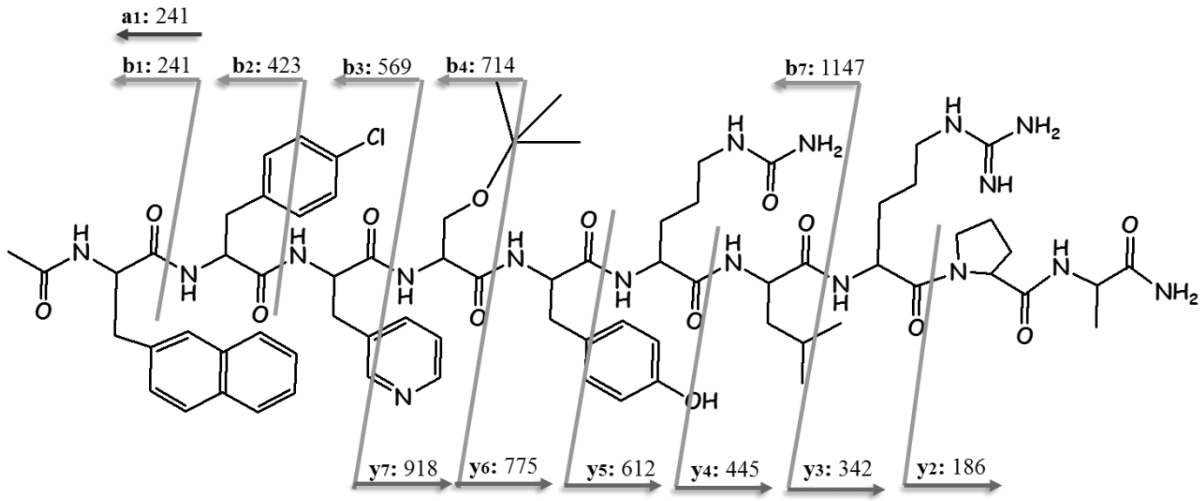

Fig. 7. Structure of impurity A and all fragment ions which were found in its product ion spectrum.

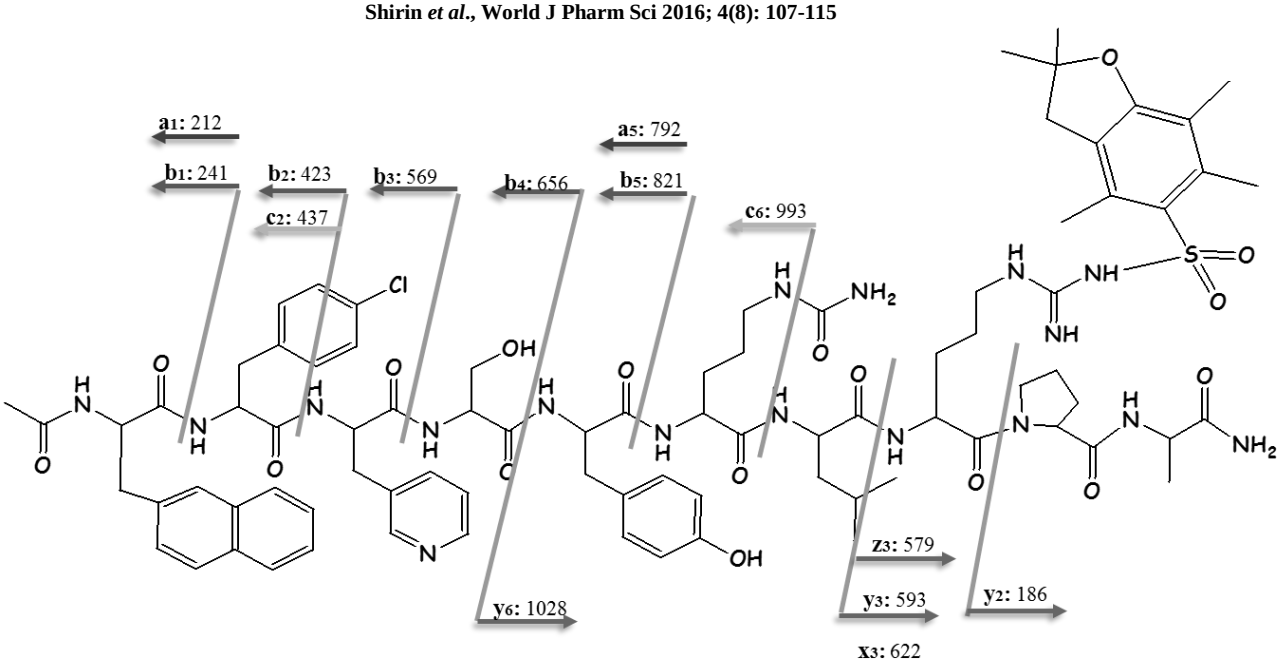

Fig. 8. Structure of impurity B and all fragment ions that were found in its product ion spectrum.

Table. 1. A summary of theoretical fragment ions of cetrorelix.

	m/z			m/z		
NO	a ions	b ions	c ions	x ions	y ions	z+1 ions
1	212	240	257	115	89	74
2	393	421	438	212	186	-
3	541	569	586	368	342	327
4	628	656	673	481	455	440
5	791	819	836	638	612	597
6	948	976	993	801	775	760
7	1061	1089	1106	888	862	847
8	1217	1245	-	1036	1010	995
9	1314	1342	1359	1217	1191	1176
10	1387	1415	-	-	1389	1374

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