Short Communication

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Identification of CJC-1295, a growth-hormone-releasing peptide, in an unknown pharmaceutical preparation

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Several peptide drugs are being manufactured illicitly, and in some cases they are being made available to the public before entering or completing clinical trials. At the request of Norwegian police and customs authorities, unknown pharmaceutical preparations suspected to contain peptide drugs are regularly subjected to analysis. In 2009, an unknown pharmaceutical preparation was submitted for analysis by liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS). The preparation was found to contain a 29 amino acid peptide with a C-terminal amide function. Based on the interpretation of mass spectrometric data, an amino acid sequence was proposed. The sequence is consistent with a peptide currently marketed under the name CJC-1295. CJC-1295 is a releasing factor for growth hormone and is therefore considered a Prohibited Substance under Section S2 of the WADA Prohibited List. This substance has potential performance-enhancing effects, it is readily available, and there is reason to believe that it is being used within the bodybuilding community. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: CJC-1295; growth hormone; doping control; Orbitrap; LC-MS/MS

Introduction

Recent advances in genomics and proteomics have led to an increased focus on the development of peptide therapeutics. Due to improved and simplified manufacturing techniques, disregard for patent protection, and a growing global market for non-approved drugs, several peptide drugs are being manufactured illicitly and are being made available to the public before entering or completing clinical trials. Some of these drugs may be misused to enhance sports performance, and thus they are prohibited by the World Anti-Doping Agency (WADA).[1]

Norwegian police and customs authorities are striving to curtail the trafficking and distribution of performance-enhancing drugs. At their request, unknown pharmaceutical preparations suspected to contain peptide drugs are regularly subjected to analysis. Over the past five years, the majority of such preparations submitted for analysis have been shown to contain human growth hormone (hGH) and the skin tanning drug Melanotan-II, respectively. Preparations containing human chorionic gonadotrophin (hCG), insulin-like growth factor-1 (IGF-1) and the growth hormone releasing peptides GHRP-2[2] and GHRP-6[3] have also been encountered by the WADA-accredited laboratories.

Experimental

All reagents used were of analytical grade and all solvents were of HPLC grade. Water was obtained using a Millipore purification system (Millipore, Bedford, MA, USA).

An unknown pharmaceutical preparation was submitted for analysis by liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS). Upon reception, the preparation consisted of approximately 2 mg of a white, flocculent powder contained in an unmarked glass vial, sealed by an aluminium cap with an orange plastic top. The vial is shown in Figure 1. An aqueous solution of the contents was prepared at a nominal concentration of 10 µg/mL. A 50 µl aliquot of the solution was diluted 1 : 9 with 50 mM aqueous ammonium bicarbonate and subjected to enzymatic digestion. Sequencing grade modified trypsin (Promega, Madison, WI, USA) was added at a 1 : 20 ratio (enzyme to protein), and the sample was incubated for 4 h at 37 °C.

The solution of the unknown substance and the tryptic digest were both subjected to analysis on a Dionex Ultimate 3000 liquid chromatography system (Dionex, Sunnyvale, CA, USA) coupled to a Thermo LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific, Bremen, Germany). The instrument system was calibrated using the manufacturer's calibration mixture, and the mass accuracy was determined to be <5 ppm during the period of analysis. A sample volume of 5 µl was injected onto the system. The chromatographic separation was performed on an LC Packings PepMap 100 C18 column (150 × 1 mm, 3 µm particle size) (Dionex, Sunnyvale, CA, USA) with mobile phases consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a total flow rate of 40 µl/min. The elution

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Figure 1. Vials found to contain hGH (left), CJC-1295 (centre) and Melanotan-II (right).

employed a linear gradient starting at 5% B, increasing to 50% B in 20 min. The mass spectrometer was operated in positive electrospray ionisation (ESI+) mode, with a spray voltage of 4 kV and a capillary temperature of 200 °C. Nitrogen was used as sheath gas at a flow setting of 8 arbitrary units. For data acquisition, a data-dependent MS/MS scan setting was applied. High resolution mass spectra were acquired in profile mode (scan range m/z 300–2000) in the Orbitrap analyzer with the resolution set at 30 000, followed by product ion scans of the two most abundant precursor ions in the linear ion trap, using a normalized collision energy of 35% and an isolation width of 3 Da.

Results and Discussion

The high resolution full-scan mass spectrum of the intact peptide, shown in Figure 2, indicates a quadruply charged ion $[M+4H]^{4+}$ at m/z = 842.4825, which corresponds to a monoisotopic molecular mass of 3365.8989 Da. The experimentally determined monoisotopic mass is consistent with the elemental composition $C_{152}H_{252}N_{44}O_{42}$ (calculated mass 3365.8936 Da, error 1.6 ppm).

Tryptic digestion yielded three peptides which were separated by liquid chromatography. The tryptic peptides were named peptide A, B, and C. The base peaks of their mass spectra were doubly charged ions $[M+2H]^{2+}$ at m/z = 667.8217 (peptide A), m/z = 493.3105 (peptide B) and m/z = 543.3355 (peptide C), respectively. The observed ion at m/z = 667.8217 of peptide A corresponds to a monoisotopic mass of 1333.6277 Da, which is consistent with the elemental composition $C_{61}H_{87}N_{15}O_{19}$ (calculated mass 1333.6303 Da, error 1.9 ppm). The product ion spectrum of m/z = 667.82 is shown in Figure 3a.

The doubly charged ion at m/z = 493.3105 of peptide B corresponds to a monoisotopic mass of 984.6054 Da, which is consistent with the elemental composition $C_{43}H_{80}N_{14}O_{12}$ (calculated mass 984.6080 Da, error 2.6 ppm).The product ion spectrum of m/z = 493.31 is shown in Figure 3b.

The doubly charged ion at m/z = 543.3355 of peptide C corresponds to a monoisotopic mass of 1084.6553 Da, which is consistent with the elemental composition $C_{48}H_{88}N_{14}O_{14}$ (calculated mass 1084.6605 Da, error 4.8 ppm).The product ion spectrum of m/z = 543.34 is shown in Figure 3c.

For each of the three tryptic peptides, a proposed amino acid sequence and a rationale for the observed fragmentation pattern is shown in Figure 4.

A discrepancy of one mass unit is observed between the mass of the intact peptide and the sum of masses of the tryptic peptides, corrected for the water added during cleavage of two peptide bonds. Comparison of the elemental compositions reveals that one nitrogen atom in the intact peptide is substituted by oxygen in

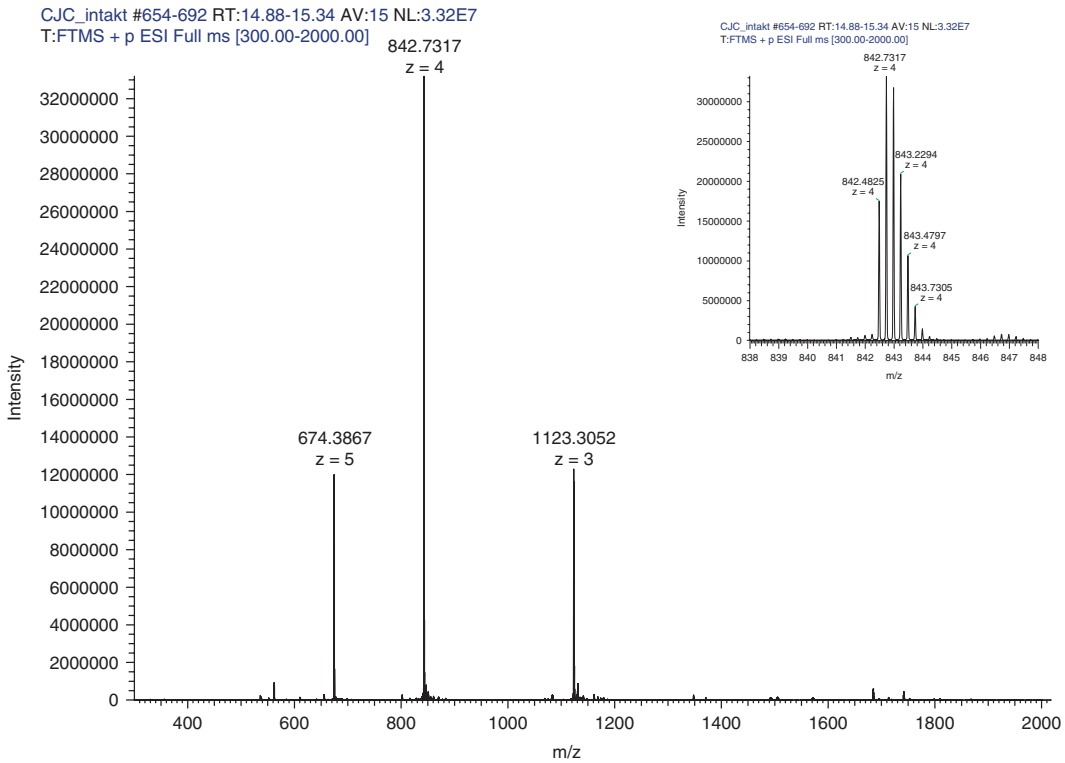

Figure 2. High resolution full-scan mass spectrum of the intact peptide found in the unknown pharmaceutical preparation.

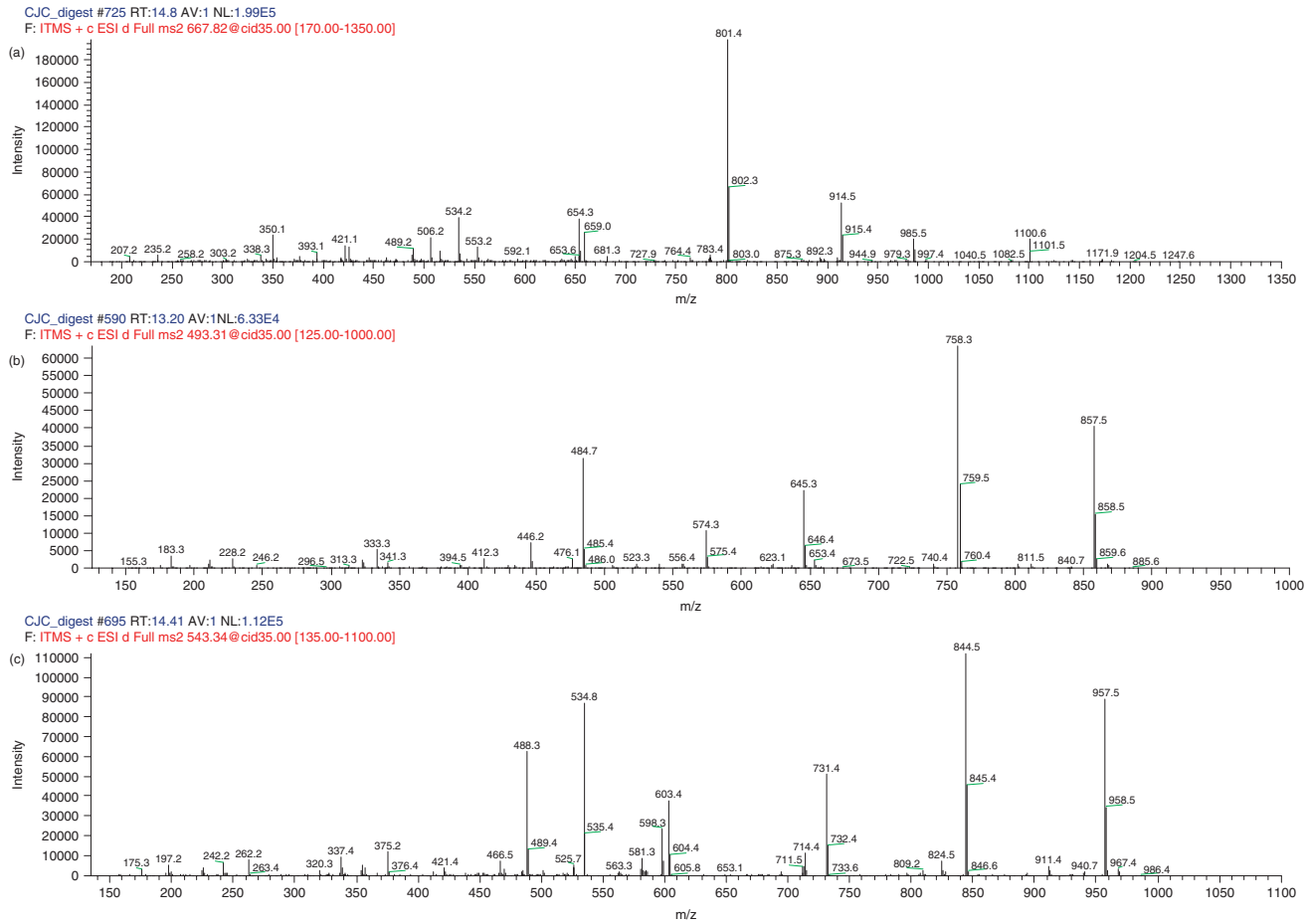

Figure 3. Product ion spectra of $m/z = 667.82$ (a), $m/z = 493.31$ (b) and $m/z = 543.34$ (c); the doubly charged ions $[M+2H]^{2+}$ of peptides A, B and C, respectively.

the tryptic digest. This indicates that a C-terminal amide function is present in the intact peptide, but not in the digest. The mass spectra of the tryptic peptides all display y_2 and y_3 fragments consistent with C-terminal carboxylic acids, confirming that deamidation occurs.

From the above results, it can be determined that the unknown substance is a 29 amino acid peptide with a C-terminal amide function. Interpretation of the mass spectrometric data leads to the proposed amino acid sequence of the intact peptide shown in Figure 5.

Tryptic cleavage occurs predominantly at positions 11 and 20, as mass the spectrometric data show that peptides B and C both contain intact N-terminal lysine residues. Although the experimental conditions appear to favour cleavage at arginine, peptides arising from alternate or additional cleavage at lysine residues were also observed at low abundance.

The amino acid sequence proposed for the unknown substance is consistent with a peptide currently marketed under the name CJC-1295. It is a synthetic 29 amino acid analogue of growth hormone releasing hormone (GHRH), with amino acid substitutions at positions 2, 8, 15 and 27.[4] It has been shown to increase serum levels of growth hormone.[5,6] Its mechanism of action is shared with GHRH and the synthetic analogues GRF_{1-29} and tesamorelin (TH9507), but it is distinct from the ghrelin class of growth hormone secretagogues.[4,7,8]

CJC-1295 was developed by ConjuChem Biotechnology Inc. (Montreal, Canada) and in its original form, it utilizes a novel bioconjugation technology referred to as Drug Affinity Construct (DAC™). A maleimidopropionamide derivative of lysine at the C-terminus allows it to bind covalently to albumin *in vivo*, thereby significantly extending its half life.[5,9] However, most products currently sold as CJC-1295, including the substance identified in this study, are non-approved drugs lacking the DAC™ feature. CJC-1295 was withdrawn from clinical trials in 2006 and is not currently approved for human use.[10]

Conclusion

An unknown pharmaceutical preparation was subjected to analysis to determine whether it contained prohibited substances. The preparation was found to contain a peptide which had not previously been encountered. The experimentally determined molecular mass and the observed fragmentation patterns are consistent with the amino acid sequence of a peptide commonly referred to as CJC-1295. Its identity could not be unequivocally confirmed, as certified reference material was not available.

CJC-1295, whether with or without the DAC™ feature, is a releasing factor for growth hormone and should therefore be regarded as a Prohibited Substance under section S2 of the WADA Prohibited List.[1] Although there is currently no indication that

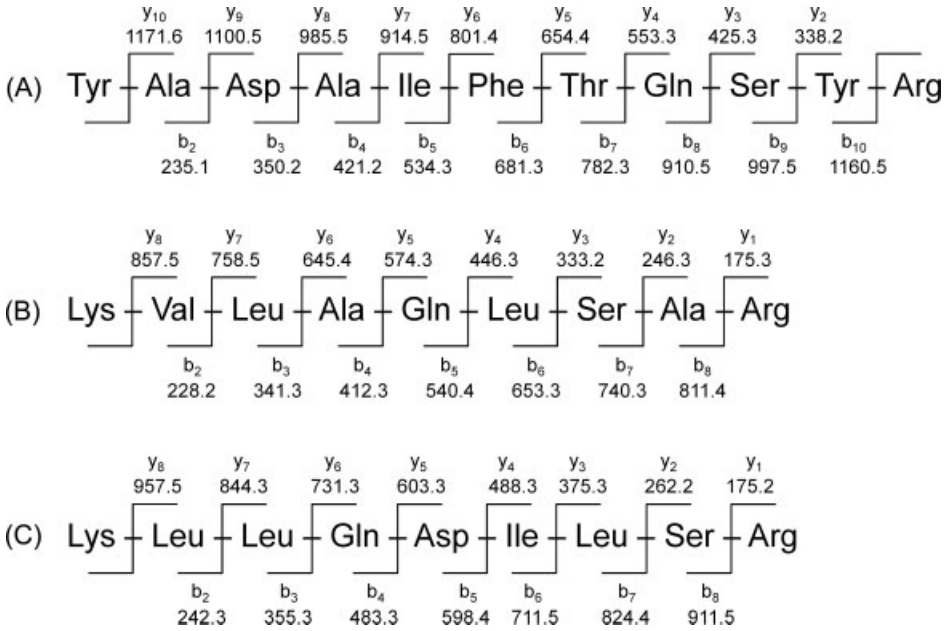

Figure 4. Proposed amino acid sequences and assignment of the fragments observed in the product ion spectra of peptides A, B, and C.

Figure 5. Proposed amino acid sequence of the intact peptide.

CJC-1295 is being misused in competitive or elite sports, it has potential performance-enhancing effects. It is readily available, as this study clearly shows, and there is reason to believe that it is being used within the bodybuilding community. The metabolism and excretion of CJC-1295 should be investigated in order to determine how its misuse best could be disclosed in sports drug testing.

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