SHORT COMMUNICATION

Identification of the growth hormone-releasing hormone analogue [Pro1, Val14]-hGHRH with an incomplete C-term amidation in a confiscated product

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ABSTRACT

In this work, a modified version of the 44 amino acid human growth hormone-releasing hormone (hGHRH(1-44)) containing an N-terminal proline extension, a valine residue in position 14, and a C-terminus amidation (sequence: PYADAIFTNSYRKVVLGQLSARKLLQDIMSRQQGESNQERGARARL-NH_2) has been identified in a confiscated product by liquid chromatography-high resolution mass spectrometry (LC-HRMS). Investigation of the product suggests also an incomplete C-term amidation.

Similarly to other hGHRH analogues, available in black markets, this peptide can potentially be used as performance-enhancing drug due to its growth hormone releasing activity and therefore it should be considered as a prohibited substance in sport. Additionally, the presence of partially amidated molecule reveals the poor pharmaceutical quality of the preparation, an aspect which represents a big concern for public health as well.

INTRODUCTION

The use of peptide-based doping agents has become increasingly popular during the last years [1]. A relevant phenomenon for this class of substances is represented by unapproved peptide formulations [2-6]. According to the Prohibited List of substances and methods of the World Anti-Doping Agency (WADA) [7], any pharmacological substance without current approval by any governmental regulatory health authority for human therapeutic use, is prohibited.

Unapproved drugs are generally not accessible through legal channels of distributions, but several cases have already demonstrated how they can find their way into black markets [2-6,8,9]. This phenomenon represents a big concern for public health, since these preparations are often prepared with no respect for good manufacturing practice, with lack or even total absence of information on composition. Moreover, these peptides do not usually undergo a complete toxicological evaluation (e.g.: TB-500) [6], and therefore the risks associated with the intake are unpredictable.

In sport drug testing, identification of new peptides with performing-enhancing properties is a first important step towards the development of effective detection methods for monitoring their misuse in sports [10,11]. In this context, collaboration between controlling authorities and laboratories has a crucial role in the battles against illegal trade of peptide hormones.

Recently, an unknown preparation, consisting of an unlabeled vial containing lyophilized powder (Figure 1), was confiscated by Belgian Customs authorities and then delivered to our laboratory for identification. The product was characterized by ultra-high performance liquid chromatography/high-resolution mass spectrometry (UHPLC-HRMS).

EXPERIMENTAL

Chemicals

Acetonitrile and water (LC grade) were purchased from BioSolve (Lexington MA, USA). Glacial acetic acid was purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Fischer Scientific (Loughborough, UK). Sequencing Grade Modified Trypsin was purchased from Promega (Madison, WI, USA). Ammonium bicarbonate was purchased by Sigma-Aldrich (St. Louis, MI, USA)

Stock solution of the unknown product was prepared by dissolving the content of the vial in water (1 mg/mL). For direct injection, sample was diluted 1:10 with 2% acetic acid.

Trypsin digestion

100 µL stock solution was evaporated to dryness (40 °C) and reconstituted in 200 µL trypsin solution (10 µg/mL in 25mM ammonium bicarbonate buffer, pH 8). Sample was incubated under gentle stirring for 24 hours at 37 °C. Trypsin digestion was stopped with 2% acetic acid (50 µL), and samples were then injected in the LC-MS system.

Liquid chromatography

The UHPLC system consisted of an Accela LC (Thermo Scientific, Bremen, Germany) equipped with degasser, Accela 1250 pump, autosampler thermostated at 10°C and a heated column compartment. LC separation was performed using a Zorbax SB-C8, 2.1 x 50 mm and 1.8 µm particle size from Agilent Technologies (Böblingen, *Germany*) thermostated at 25°C. The mobile phases were 0.2% formic acid in water (A) and 0.2% formic in acetonitrile (B). The gradient program was as

follows: 100% A for 5 min, then decreased linearly to 0% in 8.0 min, and held at 0% for 2 min followed by an increase to the initial concentration of 100% A in 0.1 min. Equilibrium time was 5 min resulting in a total run time of 20 min. The flow rate was set constant at 250 µL/min and the injection volume was 10 µL.

High-resolution mass spectrometry

HRMS characterization was performed using a Q-Exactive benchtop, Orbitrap™-based mass spectrometer (Thermo Scientific, Bremen, Germany) operated in positive-negative polarity switching mode and equipped with a heated electrospray ion source (HESI). Nitrogen sheath gas flow rate and auxiliary gas were set at 60 and 30 (arbitrary units), respectively. The capillary temperature was 350°C, the spray voltage 3 kV or -3 kV and the capillary voltage 30 V or -25 V, respectively in positive or negative ion modes. The instrument operated from *m/z* 300 to 3 000 at 70 000 resolving power in full scan (FSMS). The automatic gain control (AGC) was set to 10e6. The data acquisition rate was 2 Hz.

FSMS experiments were followed by targeted MS/MS (tMSMS) experiments at a normalized collision energy (NCE) set at 30% for peptide sequencing. For tMSMS experiments, the instrument operated from *m/z* 100 to 1 500 at 17 500 resolving power and AGC set to 10e6. Isolation window of the quadrupole was set to 3.0 *m/z*. The Orbitrap™ performances in both positive and negative ionization modes were evaluated daily and when it failed, external calibration was performed with Exactive Calibration Kit solutions (Sigma-Aldrich, and ABCR GmbH & Co. KG, Karlsruhe, Germany). A mass extraction window of 5 ppm was used.

Data analysis

ProMass software from Thermo Fisher (Bremen, Germany) was used for spectral deconvolution. Protein Prospector v 5.10.19 was used to predict both trypsin digestion products and fragmentation of peptides for de novo sequencing [12].

RESULTS

Identification of [Pro1, Val14]-hGHRH

HRMS is an ideal tool for identification of peptidic compounds due to the possibility to determine the amino acid sequence of a peptide based upon its MS/MS spectrum. Preliminary analysis constisted of direct injection for FSMS analysis. Figure 2 shows full scan FSMS spectrum of the unknown compound, which presents a cluster of multicharged ions in positive mode (typical for peptides) in the *m/z* range 500-2000, whereby the six-fold charged ion at *m/z* 872.9687 is the most abundant. Mass spectrum deconvolution yielded a monoisotopic mass 5233. 7555 Da.

In order to obtain better peptide sequencing, the peptide was incubated with the proteolytic enzyme trypsin. Tryptic digestion allowed for the formation of smaller subunits of the peptide (MW < 2000 Da) which could be easily sequenced for the identification of the intact peptide. Tryptic product peptides that were identified are all summarized on Table 1. As shown in Figure 3 for six representative peptides, de novo sequencing from MS/MS spectra of tryptic products generated by enzymatic digestion of the native peptide provided identification of the unknown substance as a 46-aa modified version of human GHRH(1-44, corresponding to the sequence PYADAIFTNSYRKVVLGQLSARKLLQDIMSRQQGESNQERGARARL and to an exact monoisotopic mass of 5233,7637 Da, confirming the value obtained from spectral deconvolution of the native peptide. The peptide has a proline residue at the N–terminus (Figure 3a and 3b), a valine residue in position 14 (Figure 3b and 3c) and

a C-term amidation which are not present in hGHRH and it will be further referred as [Pro1, Val14]-hGHRH.

The valine in position 14 was the most surprising finding, since it was inserted as an additional amino acid and not, as expectable, in substitution of an amino acid (perhaps arginine or lysine, sensible to enzymatic degradation). On the other hand, N-terminal proline extension and C-terminal amidation have been described for GHRH analogues [13,14]

Cleavage sites for [Pro1, Val14]-hGHRH were the carboxyl side of $Arg_{12,22,31,39,43}$, and $Lys_{13,23,}$ which are typical cleavage sites for trypsin. Digestion products with missing cleavages were also detected (due to incomplete digestion); however, these peptides provided further confirmation of the sequence.

C-terminus: an incomplete amidation?

Analysis of the tryptic products, particularly of the product aa 44-46, revealed the presence of both free and amidated C-terminus. As shown in Figure 4, beside the ARL-OH pseudomolecular ion (*m/*z=359.2394) (Figure 5a), the ion 358.2554 (Δ*m/z*= -0.9840) (Figure 5b) corresponding to the amidated peptide ARL-NH_2 (theoretical *m/z*=358.2561, Δppm= -1.9) was detected. This result was confirmed by the detection of the two $(y_2\text{-}NH_3)^+$ ions at *m/*z= 270.1922 (Δppm= -1.1) (Figure 4c) *m/*z=271.1758 Δppm= -2.5) (Figure 4d). The ratio between the area of the peaks indicates that the free peptide is significantly more abundant than the amidated. Unfortunately, no appropriate reference standards were available to provide a more precise determination.

As trypsin does not have deamidating activity, the free form could not be generated during the digestion. Traces of the amidated form (monoisotopic mass= 5232.7560) were also detected in the native peptide (Figure 4e and 4f).

Amidation, a common strategy to improve the pharmacokinetics properties of a peptidic drug, is generally performed in the last stages of the synthesis. Moreover, amidation is present in other GHRH analogues [4]. This finding suggests an incomplete amidation of the peptide during the manufacturing process. This is a clear example of the lack of quality controls typically associated to the production of black market peptides.

Misuse and detection of [Pro1, Val14]-hGHRH

The human growth hormone-releasing hormone (GHRH) is a 44 amino acid hypothalamic peptide (sequence: (YADAIFNTSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL) that stimulates GH production and release [14]. GHRH analogues are considered as prohibited substances according to section S2 of WADA List (peptide hormones, growth factors and related substances) [7]. To the best of our knowledge, [Pro1, Val14]-hGHRH-NH_2 has not been approved for any clinical application.

There is reason to believe that [Pro1, Val14]-hGHRH might be marketed as performance-enhancing drug. Indeed, similar to other several other analogues of GHRH(1-44) and GHRH(1-29) (tesamorelin, sermorelin, CJC-1293 and CJC-1295) [4], [Pro1,Val14]-hGHRH appears to find its way to black market and is classified by the marketers as a performance-enhancing drug. GHRH analogues appear to be attractive especially to some communities such as bodybuilding and fitness. Obviously, no pharmacokinetics data are available for [Pro1, Val14]-hGHRH, but

very low urinary and plasmatic concentrations (sub ng/mL) and short half-life can be expected. However, due to its similarity to other GHRH analogues, currently existing methods based on immunoaffinity purification followed by LC-MS detection should theoretically be able to monitor its abuse [10].

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FIGURE AND TABLE CAPTION

Figure 1

The product confiscated by Belgian Customs.

Figure 2

Full scan spectrum of the unknown peptide.

Figure 3

E MS/MS spectra for six representative [Pro1, Val14]-hGHRH-NH_2 tryptic peptides.(b-f). The C-terminus peptide ARL was detected mainly in the free form (see Figure 5).

Figure 4

Extracted ion chromatograms and spectra of $[M+H]^+$ and $(y_2\text{-}NH_3)^+$ ions from tMSMS of tryptic product aa 44-46 ARL-OH (precursor ion at *m/z*= 359.2401) (a,c) reveal the presence, in a minor amount, of 44-46 ARL-NH_2 (b,d), most likely due to an low yield of the amidation during the synthesis of the peptide. Traces of the amidated peptides were also detected in the full scan spectrum of the native peptide (e,f).

Table 1

List of tryptic peptides detected after digestion,including those with missing cleavages.