Growth Hormone & IGF Research 41 (2018) 1–6

Contents lists available at ScienceDirect

Growth Hormone & IGF Research

journal homepage: www.elsevier.com/locate/ghir

Analysis of new growth promoting black market products

Oliver Krug[a,b], Andreas Thomas[a,*], Helle Malerød-Fjeld[c], Yvette Dehnes[c], Tim Laussmann[d], Ingo Feldmann[e], Albert Sickmann[e], Mario Thevis[a,b]

[a] Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf, 50933 Cologne, Germany
[b] European Monitoring Center for Emerging Doping Agents (EuMoCEDA), Cologne/Bonn, Germany
[c] Norwegian Doping Control Laboratory, Department of Pharmacology, Oslo University Hospital, 0424 Oslo, Norway
[d] Centre of Education and Science of the Federal Revenue Administration, Cologne, Germany
[e] Leibniz-Institut für Analytische Wissenschaften-ISAS e.V., Bunsen-Kirchhoff-Str. 11, 44139 Dortmund, Germany

ARTICLE INFO

Keywords:
Mass spectrometry
Growth hormone analogue
GHRP analogue

ABSTRACT

Detecting agents allegedly or evidently promoting growth such as human growth hormone (GH) or growth hormone releasing peptides (GHRP) in doping controls has represented a pressing issue for sports drug testing laboratories. While GH is a recombinant protein with a molecular weight of 22 kDa, the GHRPs are short (3–6 amino acids long) peptides with GH releasing properties. The endogenously produced GH (22 kDa isoform) consists of 191 amino acids and has a monoisotopic molecular mass of 22,124 Da. Within this study, a slightly modified form of GH was discovered consisting of 192 amino acids carrying an additional alanine at the N-terminus, leading to a monoisotopic mass of 22,195 Da. This was confirmed by top-down and bottom-up experiments using liquid chromatography coupled to high resolution/high accuracy mass spectrometry.

Additionally, three analogues of GHRPs were identified as Gly-GHRP-6, Gly-GHRP-2 and Gly-Ipamorelin, representing the corresponding GHRP extended by a N-terminal glycine residue. The structure of these peptides was characterised by means of high resolution (tandem) mass spectrometry, and for Gly-Ipamorelin and Gly-GHRP-2 their identity was additionally confirmed by custom synthesis. Further, established *in-vitro* experiments provided preliminary information considering the potential metabolism after administration.

1. Introduction

The growth hormone releasing hormone (GHRH)/growth hormone (GH)/insulin like growth factor I (IGF-I) axis represents an effective physiological regulation process that potentially enhances the physical performance [1–5]. Thus, manifold pharmaceutical products are available which enable a manipulation of these processes at many stages. In elite sport, such manipulation is banned according to the list of prohibited substances and methods of doping [6]. Especially, the combination of the illicit use of growth promoting agents (such as GH) together with anabolic steroids were described to be very effective [3]. Most of these substances are peptide- or protein-based drugs and own the identical or closely related amino acid sequence as the endogenous counterpart. Mostly, only single amino acids are exchanged in order to enhance the stability or induce an improved efficacy profile after administration [7]. While the commercial peptide production (up to 30 amino acids) is realized by automated solid phase synthesis, proteins (such as GH) are predominantly expressed in bacterial cells (recombinant) and subsequently purified. Here, the aimed amino acid sequence is defined by the choice of the introduced vector.

In this study, products recently observed in this field with to date unknown amino acid sequences are reported. The first example is a 22 kDa recombinant GH analogue with an additional amino acid (Ala) attached to the N-terminus of the protein. In another case, different products of GHRPs were identified with an additional glycine at the N-terminus. This modification was discovered for the well-known GHRP-2, Ipamorelin and GHRP-6. Initially, GHRPs were developed as ghrelin receptor agonists to induce the GH secretion from the pituitary gland [8]. Due to their short amino acid sequence (4–6 amino acids), they are much cheaper than GH itself and some analogues (such as GHRP-2) are also orally active. Interestingly, one of these products (Gly-GHRP-6) was confiscated in Germany and Norway within a short time frame, which emphasises the international relevance of this finding. The lyophilisates were confiscated by the customs at the airport border control as imported products from Asia. Generally, these products were advertised on the internet black market to enhance muscle growth by

* Corresponding author at: Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany.
E-mail address: a.thomas@biochem.dshs-koeln.de (A. Thomas).

https://doi.org/10.1016/j.ghir.2018.05.001
Received 22 January 2018; Received in revised form 11 May 2018; Accepted 16 May 2018
Available online 19 May 2018
1096-6374/

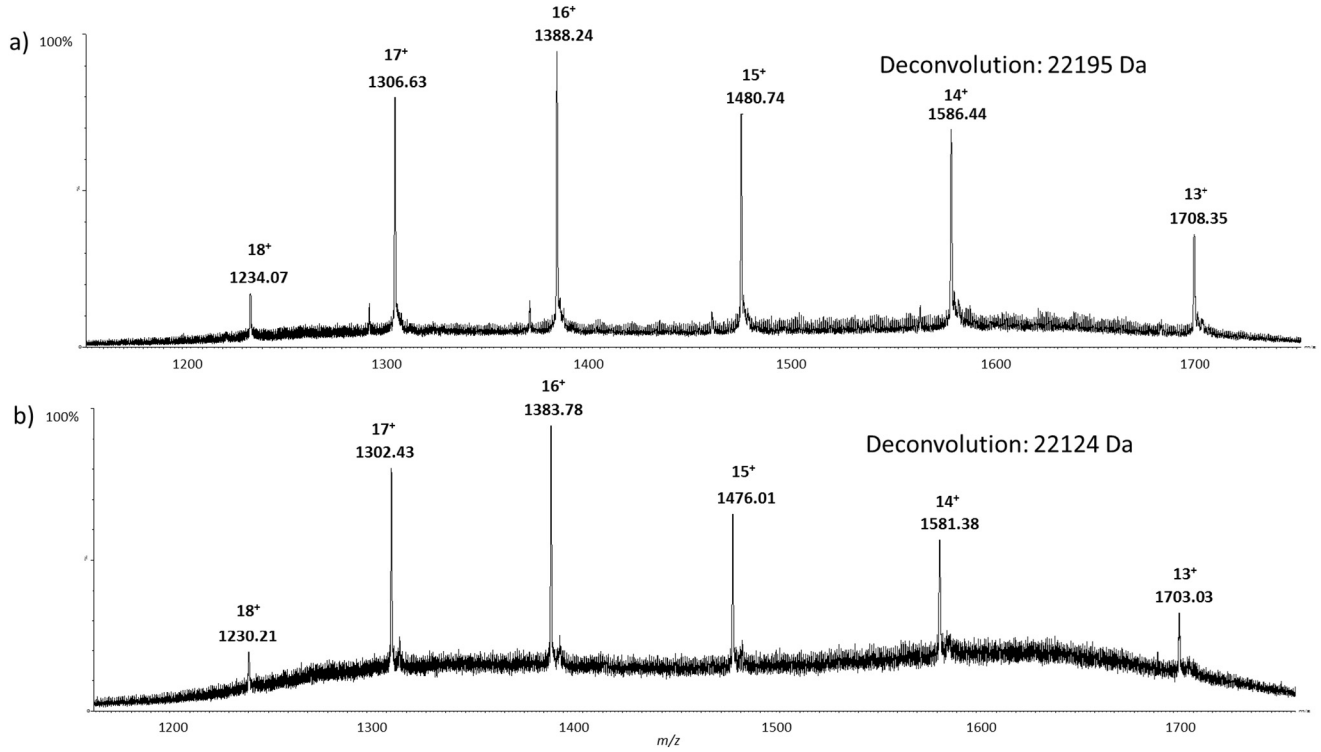

Fig. 1. High resolution full MS spectra of a) the confiscated black market product and b) the recombinant GH reference. Deconvolution yielded approximate molecular weights of 22,195 Da for a) and 22,124 Da for b).

increasing the endogenous GH/IGF production. These effects are especially (but not exclusively) desirable in the bodybuilding community.

Top-down and bottom-up analysis by means of liquid chromatography coupled to mass spectrometry represent the state-of-the-art technique for peptide or protein-based drugs. Comparable strategies were realized for GH and GHRPs before [1,9].

1.1. Chemicals and reagents

Glacial acetic acid and acetonitrile (analytical grade) were obtained from Merck (Darmstadt, Germany). Amidase as recombinant protein, expressed in *E. coli*, ≥1.5 U/mg, formic acid and phosphate-buffered saline (PBS) tablets (pH 7.4) were purchased from Sigma (Schnelldorf, Germany). Sequencing Grade Modified Trypsin was from Promega (Madison, WI). The serum specimen for the *in-vitro* experiments was from Innovative Research, Inc. (Peary Court Novi, MI). For all dilution steps and preparation of aqueous solutions, ultra-pure water was used. Peptide synthesis for Gly-GHRP-2, Gly-Ipamorelin and their stable isotopically labelled analogues (^{13}C, ^{15}N-Gly) was realized at the Leibnitz-Institute for Analytical Science (ISAS, Dortmund, Germany).

2. Black market samples

2.1. Sample preparation

The confiscated samples arrived at the laboratory as lyophilized powder in cramped injection vials. These vials were weighed and the entire content was solved in a defined volume of water. The solved samples were transferred into a fresh polypropylene tube and, after drying, the empty vials were re-weighed and the mass of the lyophilisate was determined accordingly. After appropriate dilution with water, the solutions were used for further analysis.

Bottom-up analysis was performed with an aliquot of the GH analogue by hydrolysis of approximately 200 ng of the protein diluted in 50 µL of ammonium hydrogen carbonate buffer (100 mM, pH 8) by adding 20 ng of Trypsin and incubation overnight (12 h) at 37 °C. Hydrolysis was stopped by adding 10 µL of formic acid (1%) prior to injection into the LC-MS.

In-vitro experiments were performed according to an earlier published protocol [10]. Briefly, 1 µg of each peptide was added to 100 µL of serum, 100 µL of water was added and incubated for 12 h at 37 °C under gentle shaking. Subsequently, 400 µL of acetonitrile was added and vortexed for 10 s to induce protein precipitation. After centrifugation for 10 min at 17,000 × *g*, the supernatant was transferred into a fresh tube and evaporated to dryness. The dry residue was reconstituted in 100 µL of water before injection into the LC-MS. For the incubation experiments with recombinant amidase, 1 µg of each peptide was added to 100 µL of PBS, 50 µg of amidase, 100 µL of water was added and incubated for 12 h at 37 °C under gentle shaking. Protein precipitation and all subsequent sample preparation steps were identical to the above mentioned protocol for biological fluids.

3. Liquid chromatography

Samples were injected into a LC system consisting of a Dionex Ultimate 3000 liquid chromatograph (LPG-3400XRS Pump, TCC-3000RS Column Compartment, 3x00TXRS OAS-3000 Open Autosampler) interfaced *via* electrospray ionisation (ESI) to a Thermo Fisher Scientific Q Exactive plus mass spectrometer. The aqueous solvent A was formic acid (1%) and solvent B was acetonitrile containing 1% of formic acid.

The target analytes were separated by means of liquid chromatography. The used liquid chromatographs were: Dionex Ultimate 3000 liquid chromatograph (LPG-3400XRS Pump, TCC-3000RS Column Compartment, 3x00TXRS OAS-3000 Open Autosampler) or Waters Acquity i-class UPLC (Manchester, GB). Solvents A and B for generating the gradient, formic acid (0.1%, A) resp. acetonitrile with 0.1% formic acid (B) were used. Each LC was equipped with a trapping column Accucore Phenyl/Hexyl (Thermo, Bremen, Germany) 3 × 10 mm,