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Analysis of illegal peptide biopharmaceuticals frequently encountered by controlling agencies

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ABSTRACT

Recent advances in genomics, recombinant expression technologies and peptide synthesis have led to an increased development of protein and peptide therapeutics. Unfortunately this goes hand in hand with a growing market of counterfeit and illegal biopharmaceuticals, including substances that are still under pre-clinical and clinical development. These counterfeit and illegal protein and peptide substances could imply severe health threats as has been demonstrated by numerous case reports. The Belgian Federal Agency for Medicines and Health Products (FAMHP) and customs are striving, together with their global counterparts, to curtail the trafficking and distributions of these substances. At their request, suspected protein and peptide preparations are analysed in our Official Medicines Control Laboratory (OMCL). It stands to reason that a general screening method would be beneficiary in the battle against counterfeit and illegal peptide drugs. In this paper we present such general screening method employing liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the identification of counterfeit and illegal injectable peptide preparations, extended with a subsequent quantification method using ultra-high performance liquid chromatography with diode array detection (UHPLC-DAD). The screening method, taking only 30 min, is able to selectively detect 25 different peptides and incorporates the proposed minimum of five identification points (IP) as has been recommended for sports drug testing applications. The group of peptides represent substances which have already been detected in illegal and counterfeit products seized by different European countries as well as some biopharmaceutical peptides which have not been confiscated yet by the controlling agencies, but are already being used according to the many internet users forums. Additionally, we also show that when applying the same LC gradient, it is also possible to quantify these peptides without the need for derivatization or the use of expensive labelled peptides. This quantification method was successfully validated for a representative subset of 10 different peptides by using the "total error" approach in accordance with the validation requirements of ISO-17025.

1. Introduction

Spurious/falsely-labelled/falsified/counterfeit (SFFC) medicines are a growing problem worldwide. The problem is situated both in developing countries as in industrialised regions. In the developing countries the problem concerns the whole medicine supply chain, especially essential medicines like antibiotics [1], HIV medication [2] and anti-malaria products [3]. This is often due to the lack of effective enforcement agencies and the high prices of genuine medicines in these countries. In the industrialised world the problem concerns essentially life style drugs, anabolic hormones and slimming products, although sometimes counterfeited antibiotics, insulin and anti-cancer antibodies are intercepted by the customs or controlling agencies [4]. The growing threat of these products is mainly due to the extension of the internet [5–7], where about 50% of the medicines sold through internet sites, disclosing their identity is estimated to be counterfeit [8].

Recent advances in genomics, proteomics, recombinant expression technologies and peptide synthesis have led to an increased development of protein and peptide therapeutics. Unf ortunately the uprising of these kinds of active pharmaceutical ingredients also goes hand in hand with an increase in the number of incidents with SFFC pharmaceuticals, including stolen and

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substandard proteins and peptides. Furthermore, 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 [9–18].

The Belgian Federal Agency for Medicines and Health Products (FAMHP) and customs are striving, together with their global counterparts, to curtail the trafficking and distributions of these substances. Reports originating from Germany [10,11], UK [12], Denmark [13,14], Norway [9], Italy [15] and Belgium [16–18] show that the growth hormone releasing peptides (GHRP-2, GHRP-6, CJC1295, sermorelin, Ipamorelin), Mechano Growth Factor (MGF), putative anti-obesity drugs (AOD9604), the skin tanning drug Melanotan II, the putative anti-cancer peptide epitalon and the wound healing peptide Thymosin β could be identified in seized samples. Most of the samples analysed contained lyophilised peptides with a molecular mass below 5 kDa and were upon reconstitution meant for injection purposes. It stands to reason that the battle against counterfeit and illegal peptide drugs would benefit from a general screening method for the analysis of these peptides up to 5 kDa and their subsequent quantification.

Here we present such a general screening method employing liquid chromatography–tandem mass spectrometry (LC–MS/MS) for the detection of counterfeit and illegal injectable peptides. The screening method is able to selectively identify 25 different peptides. These peptides represent illegal and counterfeited peptides encountered by the different European partners, supplemented with some peptide biopharmaceuticals which are already being used according to the different internet forums. Unfortunately the European and/or United States Pharmacopeia do not provide any guidelines for a positive identification of these substances. Therefore we applied, for the identification of these peptides, the proposed minimum of five identification points (IP) as was recommended for sports drug testing applications [19]. Furthermore, we also show that we can accurately quantify a subset of 10 different peptides representing the peptides identified in our real-life samples and having different retention times, molecular weights and chemical and biological properties by means of ultra-high performance liquid chromatography with diode array detection (UHPLC-DAD).

2. Materials and methods

2.1. Standards and reagents

The reference standards of GHRP-6 acetate (purity ≥97%), Melanotan II acetate salt (batch 091M4715V, purity ≥97%), Thymosin β4 (batch 0711538, purity ≥95%), [Arg8]-Vasopressin acetate salt (batch 011M4782V, purity ≥95%), hexarelin (batch 1432428, purity ≥90%) and Growth Hormone Releasing Factor Fragment 1-29 amide human (batch 095K4817, purity ≥97%) were purchased from Sigma-Aldrich (St. Louis, USA). GHRP-2 acetate (batch 110601, purity ≥97%) was from Chemos GmbH (Regenstauf, Germany). Buserelin (batch 2), Gonadorelin (batch 5), Leuprorelin (batch 5), oxytocin (batch 4), Protirelin (batch 3), Somatostatin (batch 5) and tetracosactide or ATCH 1-24 (batch 3) were European Pharmacopoeia reference standards. All other peptides, including Adipotide (batch 10, purity ≥98%) ATCH 1-39 (batch 10, purity ≥98%), AOD9604 (batch 10, purity ≥98%), CJC1295 without DAC (batch 10, purity ≥98%), CJC1295 with DAC (batch 10, purity ≥98%), DSIP (batch 10, purity ≥98%), GnRH Triptorelin (batch 10, purity ≥98%), Ipamorelin (batch 10, purity ≥98%), Melanotan I (batch 10, purity ≥98%), MGF (batch 10, purity ≥98%) and PT-141 (batch 10, purity ≥98%) were ordered from PepBridge Chemical LLC (Houston, TX, USA). Epitalon tetrapeptide (purity ≥99%) was purchased from Peptide Sciences (www.peptidesciences.com).

Acetonitrile was ULC-MS grade and purchased from Biosolve (Valkenswaard, the Netherlands). Water was obtained using a milliQ-Gradient A10 system (Millipore, Billerica, MA, USA). &#6; Trizma® base (batch SLBH6351V, purity ≥99.9%), Sucrose (batch SLBD2965V, purity ≥99.5%) and polyethylene glycol 4000 (batch BCBM7029V) were European Pharmacopoeia grade and purchased from Sigma-Aldrich (St. Louis, USA). Sodium chloride (batch K45393104, purity ≥99.5%) and analytical grade formic acid were bought from Merck (Darmstadt, Germany).

2.2. Sample set of suspected illegal peptide biopharmaceuticals

The sample set consisted of 65 samples which were taken by inspectors from the Belgium Federal Agency for Medicinal and Health Products (FAMHP). Collection of samples took place between 2009 and 2014. Due to the confidential nature of inspection data it is not possible to give precise information about each sample individually. Unlabelled vials, representing the vast majority of the confiscated samples, or vials labelled with the peptide name were subjected to analysis.

2.3. Sample preparation

2.3.1. Screening method

Standard stock solutions (1 mg/ml) were made in pure water and putative precipitation was removed by centrifugation for 15 min at 20238 × *g* in a microcentrifuge. For the construction of the database working solutions of 0.1 mg/ml were made in water containing 1% formic acid. For validation of the screening method, peptides were diluted into the chosen matrices (see Section 2.6).

2.3.2. Sample set

The vials containing the lyophilised powder were solubilised to a final volume of 250 μl of water supplemented with 1% formic acid. Next, the solution was subjected to centrifugation for 15 min preceding analysis by LC–MS/MS.

2.3.3. Quantification method

For the generation of the calibration curves for quantitative analysis, standard stock solutions were diluted into 6 different concentrations in 1% formic acid in water. The selected concentrations interval (100–500 μg/ml) for the validation of the quantitative method correspond to 10 to 100 times dilutions of concentrations that can often be found in these illegal and counterfeit samples.

2.3.4. Quantification of positive sample of the sample set

The reconstituted peptides (see Section 2.3.2) were either directly analysed by UHPLC or diluted with water and 1% formic acid until a concentration within the interval of the calibration line was obtained.

2.4. Instrumental conditions

2.4.1. Screening method

The acidified peptide solutions were subjected to analysis on a Dionex UltiMate 3000 Rapid Separation LC (RSLC) system (Thermo Scientific, Sunnyvale, CA, USA) coupled to an amaZon™ speed ETD mass spectrometer (Bruker Daltonics, 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 1 μL was injected