

Author's Accepted Manuscript

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PII: S0039-9140(18)30628-3
DOI: <https://doi.org/10.1016/j.talanta.2018.06.023>
Reference: TAL18765

To appear in: *Talanta*

Received date: 23 March 2018
Revised date: 6 June 2018
Accepted date: 8 June 2018

Cite this article as: Steven Janvier, Karlien Cheyns, Michaël Canfyn, Séverine Gosciny, Bart De Spiegeleer, Celine Vanhee and Eric Deconinck, Impurity profiling of the most frequently encountered falsified peptide drugs on the Belgian market, *Talanta*, <https://doi.org/10.1016/j.talanta.2018.06.023>

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Impurity profiling of the most frequently encountered falsified peptide drugs on the Belgian market

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Abstract

Advances in biotechnology and the chemical synthesis of peptides have made biopharmaceuticals and synthetic peptide drugs viable pharmaceutical compounds today and an important source for tomorrow's drugs and therapies. Unfortunately, also falsifications and counterfeit versions of these powerful and promising drugs are offered illegally via the internet. Since these falsified preparations are produced outside the legally required quality systems, end-users have no guarantee regarding the efficacy and safety of these products. Although falsified samples of biotherapeutics were already analysed, looking at a specific aspect of their quality or identity, no systematic studies have been performed regarding the presence of different impurities or possible contaminations. Therefore, in order to obtain a

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better understanding of the potential health risks related to the usage of falsified peptide drugs we performed a systematic screening of the ten most frequently encountered falsified peptide drugs on the Belgian market acquired from three different suspected illegal internet pharmacies. The screening incorporated the analysis of the active pharmaceutical ingredient (API), API-related impurities, small molecule contaminants (defined as organic small molecules not belonging to the other categories), elemental impurities and residual solvents. This comprehensive study showed that these type of illegal drugs not only have a high variation in amount of drugs per unit and a low purity (ranging between 5 and 75% for cysteine containing peptides), but also contained the known toxic class one elemental impurities arsenic (As) and lead (Pb). One sample was contaminated with Pb while multiple samples were found with concentrations up to ten times the ICH toxicity limit for parenteral drugs. Subsequent speciation of As confirmed the elevated concentrations for As and demonstrated that all As was present in the more toxic inorganic form. Together with the (sometimes) high amount of peptide impurities and the inherent dangers associated with the use of unauthorized peptide drugs (such as doping peptides or preclinical drugs) this study confirms the reported potential health risks patients/users take when resorting to falsified peptide drugs. Moreover, the presence of the carcinogen As and the known accumulation in human tissues of Pb raises questions about potential sub-acute to chronic toxicity due to the long term administration of these falsified peptide drugs.

Keywords: Falsified medicines; Peptide drugs; Heavy metals; Impurity profiling; Mass spectrometry

1. Introduction

Biopharmaceuticals and synthetic peptide drugs have acquired a substantial market share in the pharmaceutical sector over last two decades¹. Inevitably, also falsifications of these powerful and promising drugs have proliferated across the globe. Falsified medicinal products, defined by the WHO in May 2017 as falsifications of authorized and unauthorized medicinal products that deliberately/fraudulently misrepresent their identity, composition or source, encompassed mainly lifesaving medicines such as anti-malaria drugs in the developing world and life style drugs such as PDE 5 inhibitors in the developed world²⁻⁴. Recently however, also falsified biopharmaceuticals and synthetic peptide drugs have found

their way to the (black) market due to the expansion of the internet, economic globalization and advances in biochemistry and knowledge of human physiology. Although the vast majority of these falsified biotechnology drugs comprise life style drugs such as doping peptides, putative anti-obesity drugs and skin tanning peptides, also medicines such as neurohormones (e.g. oxytocin), insulin, human chorionic gonadotropin, human growth hormone (hGH), growth factors and monoclonal anti-cancer antibodies are prone to falsifications⁵⁻¹⁰. Since these falsified preparations are produced outside the legally required quality systems, end-users have no guarantee regarding the efficacy and/or safety. Multiple literature reports have already shown that these deplorable practices can result into serious health issues in addition to the resultant infringements on intellectual property rights and significant economic losses¹¹⁻¹⁴. These preparations are often found to contain no active pharmaceutical ingredient (API), the wrong API or a wrong dosage^{7,15,16}. Additionally, numerous biotechnology drugs which are still subject to functional or toxicological assessments or which haven't been authorized, due to either lack of efficacy or adverse side effects during pre-clinical or clinical trials, have been introduced on the illegal market via internet. There, they are often presented as genuinely efficient and safe drugs, thereby misleading end-users/patients about the safety and efficacy of these unauthorized drugs¹⁷⁻²⁰. In worst cases, they are found to contain, whether or not synthesis related, toxic (chemical or biological) contaminants or impurities^{10,21,22}.

Although falsified samples of biotechnology drugs were already analysed, looking at a specific aspect of their quality or identity^{6,7,18,23-27}, no systematic studies have been performed on falsified biotechnology drugs regarding the presence of different impurities either derived from the API during synthesis or degradation, or as a result of possible contaminations. Multiple reports in literature have shown that synthesis related impurities, elemental impurities and residual solvents or non-drug related chemical compounds (such as

glass particulates, diethyl glycerol, methyl chloride,...) are a major safety issue in counterfeit/falsified 'small molecule' medicinal products, which even in certain cases lead to deadly outcomes^{28,29}.

Therefore, in order to obtain a better understanding of the potential health risks related to the usage of falsified biotechnology drugs we performed a systematic screening of the ten most frequently encountered falsified biotechnology drugs on the Belgian market⁶. These selected biotechnology drugs encompass the tetrapeptide epitalon, the growth hormone secretagogues GHRP-2, GHRP-6 and ipamorelin, the neurohormone oxytocin, the skin tanning peptide melanotan II, the wound healing peptide thymosin β 4, the hGH-analogue AOD-9604 and the human growth hormone releasing hormone analogues (hGH-RH-analogues) sermorelin and CJC-1295. The latter peptide is also available in a conjugated form with maleimidopropionic acid linked to the ϵ -amino group of the C-terminal lysine, called CJC-1295 DAC. The selected peptide for this study however is CJC-1295 without DAC, referred to in this manuscript as 'CJC-1295'.

Seen the unique synthesis processes to obtain these biologically active peptides, different types of impurities or contaminations might be present compared to traditional small molecules. Synthesis routes depend namely on the size, amino acid sequence and the presence of modifications (incorporation of D-amino acids and/or non-canonical amino acids or post translational modifications (PTMs),...). Peptides (generally < 50 amino acids) are mainly produced by chemical synthesis, as described elsewhere^{30,31}, whilst proteins (with or without PTMs) are generally produced via recombinant expression technology or obtained via native protein isolation. Except for the actin sequestering peptide, thymosin β 4 (43 amino acids), all selected peptides have a molecular weight lower than 5 kDa and most of them (GHRP, Melanotan II,...) contain also D-amino acids and/or non-canonical amino acids. Only for thymosin β 4 both the chemical synthesis and recombinant production via

Escherichia coli have been described in literature³²⁻³⁵. Nonetheless, it is unlikely that the latter would be preferred due the efficiency and putative automatization of peptide synthesis techniques (solid phase or solution phase peptide synthesis and chemical peptide ligation). Therefore it is a relatively safe presumption that all investigated peptides were synthesized via chemical synthesis and are not a product of recombinant expression technology or of biological origin.

Expected impurities that can arise from these synthesis techniques are API-related impurities such as truncations, insertions or deletions of amino acids, modifications of functional groups, incomplete deprotection, oxidations or reductions of functional groups (e.g. cysteine) and formation of aggregates³⁶. Furthermore, residual solvents and reagents or scavengers used during synthesis might remain in the peptide preparation whilst also metals (sometimes used in oxidation/reduction reactions) and metalloids may be used during peptide synthesis or originate from contaminations.

Therefore, based on pharmacopeial texts and ICH guidelines on peptide impurity profiling and the expected impurities, we screened the above mentioned set of peptides for API-related impurities, small molecule contaminants (defined as organic small molecules not belonging to the other categories), elemental impurities and residual solvents³⁷.

In order to investigate the putative presence of these impurities or contaminants, both reversed phase liquid chromatography (RP-LC) and hydrophilic liquid interaction chromatography (HILIC) were used in combination with ion trap mass spectrometry (IT-MS) and (high resolution) time-of-flight mass spectrometry (TOF-MS) for the detection and identification of all API-related impurities and ultraviolet spectroscopy for the semi-quantification of all API-related impurities. Additionally, potential small molecule contaminants were assessed with RP-LC-IT-MS and gas chromatography coupled to single quad mass spectrometry (GC-MS) whilst elemental impurities and residual solvents were

analysed by respectively inductively coupled plasma mass spectrometry (ICP-MS) and head space gas chromatography hyphenated to a single quad mass spectrometer (HS-GC-MS).

2. Material and methods

2.1 Reagents and standards

2.1.1 Reagents

Methanol (HPLC-grade) and acetonitrile (HPLC and MS-grade) were purchased from Biosolve (Valkenswaard, the Netherlands). Formic acid (ACS and Ph.Eur. reagent) and dimethyl sulfoxide were purchased from Merck (Darmstadt, Germany). Aqueous ammonium formate (10 M solution, ACS reagent) was obtained from Sigma Aldrich (St. Louis, USA). A MilliQ-Gradient A10 system (Millipore, Billerica, MA, USA) was used to prepare deionized water ($\rho=18\text{ M}\Omega\text{ cm}$, $\text{TOC} < 4\text{ppb}$).

2.1.2 Standards

Certified reference standards of GHRP-6 acetate (batch BCBM2185V, purity $\geq 97\%$), ipamoreline acetate (batch 065M4736V, purity $\geq 99.6\%$), melanotan II acetate salt (batch 091M4715V, purity $\geq 99\%$) and growth hormone releasing factor 1-29 amide human (batch LI0776, purity $\geq 98\%$) were purchased from Sigma Aldrich (St. Louis, USA). GHRP-2 (batch hor271b, purity 98%) was purchased from Prospec-bio (Rehovot, Israel). Oxytocin (batch 5.0, purity 98%) and leuprorelin (batch 5.0, purity 99%) were European Pharmacopoeia reference standards (Strasbourg, France).

Epitalon (batch 100611.1, purity $> 98\%$), AOD-9604 (batch 100611.2, purity $> 95\%$) and CJC-1295 without DAC (batch 100611.3, purity $> 97\%$) were custom synthesised by Thermo Fisher Scientific (Waltham, USA). Analytical grade mannitol (lot 13A14-B02-281665) was acquired via Fagron (Nazareth, Belgium).

The elemental stocksolutions CZ9090 for elements As, Cd, Co, Cr, Cu, Li, Mo, Ni, Pb, Se, Tl, V, Zn, IV-stock-38 for elements Ir, Os, Pd, Pt, Ph, Ru and TraceCERT mono element solutions of Ag, Au, Sn and Sb were acquired from respectively from Analytika (Prague, Czech Republic), Inorganic Ventures (USA) and Sigma-Aldrich (USA).

2.2 Sample set

The sample set was composed of 27 illicit peptide preparations which encompassed the ten most frequently encountered peptide drugs on the Belgian market acquired via three different suspected illegal internet pharmacies (see Table 1). All 27 acquired peptide preparations were delivered in glass vials containing lyophilized powder and were meant for subcutaneous injections. Different vials of the 27 samples were used for each respective analysis.

2.3 Identification of the API and API-related impurities

2.3.1 Sample preparation

Native peptides

Stock solutions of all samples were prepared by adding 1.0 ml of water and stored at minus 20°C for maximum one year. For the analysis with RP-LC-MS subsequent dilutions were made with water and formic acid (FA) to obtain individual solutions of 0.1 mg/ml 1% (v/v) FA based on the claimed peptide amount. Dilutions for the HILIC-MS analysis were made with acetonitrile (ACN), water and 1.0 M ammonium formate pH 3.0 to obtain solutions with a concentration of 0.1 mg/ml in 10mM ammonium formate 80% (v/v) ACN.

Tryptic digests

Enzymatic digestions were obtained with sequencing grade trypsin (Promega, Madison, WI, USA) for the peptides AOD-9604, sermorelin, CJC-1295 and thymosin β 4. Concretely, 50 μ g

of peptide in 100 μ l 50 mM ammonium bicarbonate (AMBIC) was subjected to a reduction step of 30 minutes at 60°C with 1 μ l 1 M dithiothreitol (DTT) and subsequently alkylated with 1 μ l 1M iodoacetic acid (IAA). The latter reaction, performed in the dark, was quenched after 30 minutes by adding 2 μ l DTT. The actual digestion was performed overnight at 37°C by adding 5 μ l of a 0.2 μ g/ μ l trypsin solution and 100 μ l of water. After ending the enzymatic reaction by adding 2 μ l FA all solutions were evaporated by placing them at 30°C for 5 hours in a rotary evaporator (Concentrator plus, Eppendorf AG, Hamburg Germany). Samples were then dissolved again in 100 μ l water containing 1% FA and centrifuged for 15 minutes with a force of 20 000 x g. Since not all peptides contained disulfidebridges, tryptic digests were performed with and without the reduction step.

2.3.2 Instrumentation

RPLC-IT-MS and HILIC-IT-MS

Analysis of the API and API-related impurities via UHPLC-IT-MS was performed using a Dionex Ultimate 3000 Rapid Separation LC (RSLC) system (Thermo Scientific, Sunnyvale, CA, USA) hyphenated to an amaZon™ speed ETD mass spectrometer (Bruker Daltonics, Bremen, Germany). Conventional reversed phase separation was achieved on an Acquity UPLC CSH C18 column (2.1 mm x 150 mm, 1.7 μ m) (Waters Milford, MA, USA) with a total run time of 30 minutes at a flowrate of 0.3 ml/min as described by Vanhee *et al* (2015). Briefly, mobile phase A and B consisted of respectively water and ACN both acidified with FA to 0.1% v/v. The sample compartment was kept at 15°C while the injection volume was set to 1 μ l. Elution was performed at 40°C by employing a multistep gradient comprised of an initial isocratic step at 2% B for one minute, a two-stage linear gradient (to 24% B in 11 minutes and subsequently an increase to 30% B in 8 minutes) followed by a steep increase to

98% B in 0.5 minutes, an isocratic washing step for 3.5 minutes and a 5 minute re-equilibration step at 2% B.

HILIC chromatography was performed at a flow rate of 0.27 ml/min and a column temperature of 45°C with a ZIC[®] HILIC (100 mm x 2.1 mm, 3.5µm) (Merck, Darmstadt, Germany) in a total run time of 35 minutes with the same LC instrumentation as used for RPLC-IT-MS²⁴. Mobile phase A and B consisted of respectively 80:10:10 (v/v) and 10:80:10 (v/v) ACN:water:100 mM ammonium formate pH 3.0. The employed gradient consisted of an isocratic elution for 2 minutes at 100% A followed by a linear decrease to 40% A in 15 minutes and 3 minutes isocratic elution prior to an online cleaning step made up of a further linear decrease to 20% A and subsequent 3 min isocratic rinsing before returning to initial conditions. The required post-gradient equilibration was performed by maintaining the initial conditions for 10 minutes. Temperature of the sample manager and injection volume were set to respectively 15°C and 4 µl.

Ion trap mass spectrometry was performed both for RP-LC as for HILIC chromatography with electrospray ionization (ESI) operating in positive mode at maximum resolution. The capillary and endplate voltage were respectively set to 4500 and 500 V while the nebulizer, desolvation temperature and desolvation gas flow were operated at respectively 2 bar, 250°C and 10 l/min. MS-spectra were recorded within a range of 200 to 1400 m/z with the smart parameter setting (SPS), ion charge control (ICC) and maximum accumulation time in the trap were set to 750 m/z , 190 000 and 50 ms. Subsequent fragmentation was performed by means of collision induced dissociation (CID) with helium as collision gas and selecting the most intense ions in the respective MS-spectra (absolute threshold: 25 000, relative threshold: 5%) with the ICC set to 200 000 and 100 ms as maximum accumulation time. Fragmentation was performed at 100% amplitude in maximum 32 ms with the Smart Frag[™] Enhancer range for ramping set from 80 to 120%.

RPLC-IT-MS of tryptic digests

All tryptic digests were analysed with the same instrumentation and mobile phases as used for the analysis of the native peptides with RPLC-IT-MS. The employed gradient however consisted of an isocratic step at 2% B for 1 min, followed by a linear gradient starting at 2% B, increasing to 50% B in 60 min prior to column washing for five minutes at 99% B and a re-equilibration step of 2 minutes at 2% B, as described elsewhere²³. The injection volume was set to 2 μ l, all other parameters were equivalent to the settings used for the analysis of the native peptides. The ion trap mass spectrometer also operated with the same setting as for the analysis of the native peptides with the exception of the mass range which was lowered to a mass range of 200-1200 m/z . Single charged ions were included considering the low mass and charge state of the respective peptides.

RPLC-Q-TOF HRMS

High resolution mass spectrometry experiments were performed by means of a Waters Synapt G2Si (Milford, MA, USA). Chromatographic separation was achieved by means of an Acquity UPLC system (Waters, Milford, MA, USA) consisting of a binary solvent manager and a sample manager with the same column, mobile phases, gradients and settings as used for the analysis of native peptides with RPLC-IT-MS. The Synapt Q-TOF system was operated in ESI positive and high resolution mode (40 000 full with at half maximum). Acquisitions were performed within a range of 50 to 2000 Da in MS^E with continuum data format. Capillary and sampling cone were operated at respectively 3.0 kV and 30 eV while the cone gas flow and source temperature were set to respectively 30 l/h and 120°C. Desolvation gas flow, desolvation temperature and nebulizer gas flow were set to 800 l/h, 450°C and 2.5 Bar. During the analysis leucine enkephalin (1 ng/ μ l) was infused at a rate of 5

$\mu\text{l}/\text{min}$ and a lockspray voltage of 2.5 kV. Corrections via lockspray were applied during analysis. MS^{E} was performed by ramping the voltage in the transfer cell from 10 to 45 eV.

2.4 Semi-quantification of API-related impurities via UV

2.4.1 Sample preparation

Semi-quantification of the API-related impurities was performed with three fold dilutions with ACN and 1% FA for HILIC-DAD analysis and with undiluted stock solutions acidified to 1% FA for analysis with RP-LC-DAD. Prior to injection, all obtained solutions were centrifuged at 20 000 x g for 15 minutes.

2.4.2 Instrumentation

Chromatographic separation was achieved with the same instrumentation as mentioned in section 2.3.2 for RP-LC-IT-MS and HILIC-IT-MS for the tetrapeptide epitalon. The injection volume was 10 μl , except for the peptides oxytocin and AOD-9604 (5 μl). UV chromatograms were recorded at two different wavelengths namely 214 nm and 280 nm. The wavelength at 214 nm was used for quantification purposes. All detected peaks with a signal to noise above 3.3 were selected and integrated. To circumvent errors due to saturation, the relative amount of the respective API was determined at a concentration of 0.3 mg/ml for each sample. Subsequently, the relative amounts of each detected API-related impurity and the LC-purity was calculated by employing the following formulas:

$$LC - Purity = \frac{AUC_{API,c=0.3 \text{ mg/mL}} * DF}{AUC_{API,c=0.3 \text{ mg/mL}} * DF + \sum_{i=1}^n AUC_{Impurity,i}}$$

$$\text{Relative Area Impurity, } k = \frac{AUC_{\text{impurity},k}}{AUC_{\text{API},c=0.3 \text{ mg/mL}} * DF + \sum_{i=1}^n AUC_{\text{impurity},i}}$$

With $AUC_{\text{API},c=0.3 \text{ mg/mL}}$ the area of the API based on the chromatogram of the respective peptide preparation at a concentration of 0.3 mg/ml, DF the dilution factor between the concentration used for the analysis of the API-related impurities and the concentration of 0.3 mg/mL used to determine the area of the API, n the number of API-related impurities and k random number between 1 and n of the respective sample. Both the solvent peak and the peak corresponding with mannitol were not included in the final calculation of the LC-purity.

2.5 LC-MS screening for small molecule contaminants

2.5.1 Sample preparation

For the screening on small molecule contaminants 2.0 ml of MeOH was added to all 28 preparations. After 30 seconds of vigorous vortexing all vials were centrifuged for 5 minutes to separate the supernatant from putative precipitate. The obtained pellet was dissolved in 1.0 ml of deionised water after an overnight drying step at room temperature. Both the supernatant and the dissolved pellet were centrifuged with a microcentrifuge for 15 minutes at 20 000 x g before the analysis via LC-MS. See supplemental figure 1 for a general schematic for the analysis of small molecule impurities.

2.5.2 Instrumentation

Both the solubilised pellet and supernatant were injected on the previously mentioned UHPLC-IT-MS system. The screening was performed based on RP-LC by means of a Waters BEH C18 (100 mm x 2.1 mm, 1.7 μm) operating at flow rate of 0.5 ml/min and a 14 minute gradient consisting of a linear increase in 9 minutes from 1 to 99% B followed by a 2 minute

isocratic rinsing step before returning to initial conditions in one minute and a one minute isocratic post-gradient equilibration step³⁸. Mobile phase A and B, injection volume, column and sample manager temperature were equal to the conditions used for the RP-LC analysis described in section 2.3.2. Detection via IT-MS was performed in ESI mode with alternating polarity at ultra-scan rate. Capillary and end plate voltage were set to respectively 3500 and 500 V. The nebulizer was set to a pressure of 3.0 bar while the desolvation gas flow and temperature were respectively 12.0 l/min and 300°C. MS-spectra were recorded between 100 to 950 m/z with the SPS, ICC and max accumulation time respectively set to 475 m/z , 100 000 and 200 ms. The most intense ions above the absolute intensity threshold of 2500 and 5% relative intensity threshold were selected for MS²-fragmentation via CID with helium as collision gas. Fragmentation amplitude and fragmentation time were set to respectively 100% and 20 ms while the SmartFrag™ Enhanced for amplitude ramping ranged from 75 to 150%. The acquired LC-MS and LC-MS² spectra were interpreted via Compass® Data Analysis 4.2 software and compared with an in-house made library³⁸.

2.6 GC-MS screening for small molecule contaminants

2.6.1 Sample preparation

The same sample set and sample preparation was used as described in section 2.4.1. The obtained supernatant after the extraction with 2.0 ml MeOH was subsequently filtered with a 0.45 µm PTFE filter prior to injection in the GC-MS system (see supplemental Figure 1).

2.6.2 Instrumentation

GC-MS analysis was performed as described by Vanhee *et al.* (2018) with an Agilent Technologies 7890A GC hyphenated to an Agilent 5975C single quad mass detector. Samples were injected (split ratio 50 to 1) at 300°C and separated with an Agilent capillary

HP-5MS column (30 m x 0.25 mm, 0.25 μ m) and helium as eluent at an isobaric pressure of 74.076 kPa³⁸. The employed two-stage temperature gradient consisted of an isothermal step for 2 minutes at 80°C before ramping to 280°C in 13.33 min and an isothermal step at 280°C for 15 minutes followed by a second ramping in 3 minutes to 310°C and holding the acquired temperature for 20 minutes. Detection via single quad MS was performed by electron impact ionisation and measuring in full scan mode (25 to 600 m/z). Source and single quad temperature were respectively set to 230°C and 150°C.

2.7 ICP-MS analysis for elemental impurities

2.7.1 Sample preparation

For the analysis of elemental impurities minimum 2.0 mg of each individual peptide preparation was accurately weighed in a plastic digiprep tube (SCP Science, Canada). When necessary, two vials of the same peptide and same vendor were pooled to obtain the required minimum of 2.0 mg of sample. Subsequently, ten minutes after the addition of 0.5 ml nitric acid, 0.125 ml HCl and 0.25 ml H₂O₂ were added to each tube. After ten minutes the samples were further diluted with 1.75 ml bidistilled H₂O. Subsequent digestion was performed by placing the tubes in a heating block (digiprep, SCP Science, Canada) at 25°C for 14h after which the temperature was ramped to 90°C in 1h and maintained for 7h at 90°C. After digestion, all samples were further diluted to a final volume of 25 ml before ICP-MS analysis.

For the subsequent speciation analysis of arsenic (As) the contents of a vial was weighted in a 15 ml tube. After addition of 1.8 ml 0.11 M HNO₃ and 0.2 ml H₂O₂ (30%), the samples were extracted for 1 hour at 90°C in a hot water bath. The combination of the weak acid with the H₂O₂ added causes arsenite (As^{III}) to oxidate to arsenate (As^V), which can be quantified as the total amount of inorganic As (As_i). Extracts were centrifuged (10 minutes, 12 500 x g) and

filtrated (0.45 μm) before analysis. The reference material NMIJ 7532a (brown rice flour; As_i = 0.298 ± 0.008 mg/kg, National Metrology Institute of Japan), was added in duplicate to the batch.

2.7.2 Instrumentation

Most element measurements were carried out with an Agilent 8800 triple quadrupole ICP-MS instrument (Agilent Technologies, USA). This instrument contains an octopole-based collision/reaction cell, located in-between two quadrupole analyzers. Possible interferences on most elements were removed using He as collision gas in the octopole. For the analysis of As and Se, O_2 was used in the octopole instead of He. To account for possible matrix induced interferences, an internal standard containing Sc and In was added in-line. Lead analysis was performed on a Varian 820 ICP-MS (Varian, Australia), with H_2 as reaction gas to obtain the desired LOQ values. Mercury was analysed in 100 μl of the digested solutions using an Advanced Mercury Analyzer (AMA 245, PS analytical, France).

Quantification of elemental impurities was performed by external calibration (calibration range: 0.2 $\mu\text{g/l}$ - 10 $\mu\text{g/l}$) using acidified (2% HNO_3 / 0.5 % HCl) dilutions of (multi)-element stock solutions: CZ9090 for elements As, Cd, Co, Cr, Cu, Li, Mo, Ni, Pb, Se, Tl, V, Zn from (5% HNO_3), IV-stock-38 for elements Ir, Os, Pd, Pt, Ph, Ru (15% HCl) and TraceCERT mono element solutions of Ag, Au, Sn and Sb (2% HNO_3). An overview of analysed masses, reaction cell modes and internal standards used is given in Supplemental Table 1. The limit of quantification of the method was determined as 10 times the standard deviation of 20 procedure blanks (acid with no sample). The final LOQ was calculated with the respective dilution factor depending on the amount of sample available. Trueness and repeatability of the method were determined after spiking of the sample with stock solutions. The samples (each time 2 mg) were spiked at three concentration levels: 0.5, 5 and 10 mg/kg. Only the

concentration levels higher than the appropriate LOQ were taken into account. No matrix relevant certified reference material was available, but the extraction efficiency was determined based on Cd (1520 µg/kg), Co (570 µg/kg) and Cu (4700 mg/kg) concentrations in reference material Tomato leaves NIST 1573a (NIST, USA).

The analysis of As species was performed using HPLC-ICP-MS (high performance liquid chromatography – inductively coupled plasma – mass spectrometry; Varian, Mulgrave, Australia). Chromatographic separation of the As species was performed with isocratic elution on an PRP-X-100 Hamilton anion exchange column with 40mM ammoniumcarbonate at pH 9.4 (1 ml/min, 60 µl injected). The chromatographic software (GALAXY) of the ICP-MS instrument was used for quantification of the peak area. A five point external calibration (0-5 µg/l) with the standard compounds As^V, Methylarsonate (MA), Dimethylarsinate (DMA) and arsenobetaine (AB) was carried out.

2.8 HS-GC-MS analysis for residual solvents

2.8.1 Sample preparation

The content of all 28 vials was suspended by adding 1.0 ml of distilled water. Subsequently, 500 µl was accurately transferred in a 10 ml head space vial for quantification of the potential residuals solvents and electronically sealed. The remaining amount of approximately 500 µl, transferred in another 10 ml headspace vial, was used for the initial screening step. All samples were stored in sealed headspace vials at 4°C for maximum one month.

2.8.2 Instrumentation

The analysis of residual solvents was based on the European Pharmacopeia and an earlier in-house validated method³⁹. All samples were injected via an G188A static headspace sampler and analysed via an 6890N GC hyphenated to a 5973N single quad mass selective detector

(all from Agilent Technologies, Palo Alto, USA). Prior to injection into the GC-MS system, samples were agitated and subjected to 120°C for 30 minutes to generate the required vapour phase. After incubation, 0.5 ml of the ambient vapour phase was injected via split injection (ratio 10:1) into the GC-MS system while temperature of the headspace loop and transfer line were maintained at respectively 110 and 115°C. Subsequent chromatographic separation was achieved in 23.4 minutes on Agilent Varian CP-Select 624 CB (60-m x 0.32 m; 1.8 µm film thickness) employing helium as carrier gas (0.806 bar) and a temperature gradient composed of an initial isothermal step of 5 minutes at 60°C before ascending (rate: 25 °C/min) in 13.4 minutes to 270°C and a second isothermal elution step at 270°C for ten minutes. Eluting compounds were subsequently ionized via electron impact (70 eV) at a source temperature of 230°C. Screening of fragments was performed with a single quad at 150°C measuring in a mass range from 25 to 400 *m/z*. Positively identified residual solvents were quantified via single ion monitoring (SIM) with the respective quantification and qualification ions (see supplemental Table 2). Quantification was performed by injecting all samples in series with a four point calibration curve (70, 90, 110 and 130) and a quality control (100%) based on the estimated concentration in the initial screening. The initial estimation was performed by one point calibration via the 100 ppb standards used to conform the identity of the identified residual solvents. For each calibration curve, the coefficient of determination and quality coefficient were calculated while the quantification results had to fall within the range of constructed calibration curves.

3. Results

The analyzed peptide set contained the ten most frequently encountered falsified peptide drugs that have been confiscated during seizures by Belgian regulatory agencies between 2009 and 2017. From those peptides, only oxytocin and sermorelin have acquired approval as

medicines. The latter was approved in 1997 for the treatment of idiopathic growth hormone deficiency in children with growth failure and as diagnostic for the hypothalamus-hypophysis axis. Both the medicine and diagnostic were however withdrawn at request of the manufacturer in 2009 and are currently categorized as ‘Discontinued’⁴⁰. The manufacturer stated these products were not withdrawn from sale for reasons of safety or effectiveness. The neurohormone oxytocin is currently used to induce labor, to control the contractility of the uterus and postpartum haemorrhages and as adjunctive therapy to manage incomplete/inevitable/elective abortion. In 2014, it acquired the orphan drug status in Europe for the treatment of Prader-Willi syndrome. In addition, GHRP-2 is currently only used as diagnostic agent in Japan to assess the functionality of the hypothalamus-hypophysis axis whilst AOD-9604 has the “Generally Recognised as Safe” (GRAS) status in the USA.^{41–43} All other peptides were either never submitted to clinical trials, are still the subject of clinical trials or were subjected to clinical trials as medicine but were halted owing to a lack of efficacy or safety (CJC-1295, ipamorelin). Except for oxytocin and melanotan II, all peptides are registered on the list of the World Anti-Doping Agency (WADA)⁴⁴.

All 27 illicit preparations were acquired as ‘research chemicals’ via three suspected illegal internet pharmacies and delivered in sealed plastic bags or small carton boxes enclosed in an envelope without any additional information (certificates, storage and handling recommendations). All peptide preparations were delivered as lyophilized powder in glass vials (resembling aseptically closed vials) which were labelled with the claimed peptide and respective amount. Only one of the vendors (vendor X) also displayed a batch number on the label. This batch number however did not differ between different peptides nor between vials of the same peptide. Moreover, there was a large variability in the amount of lyophilized material present between vendors and between samples of the same vendor. This visual

observation was corroborated by the measured weights as displayed in table 2 (e.g. epitalon, AOD-9604).

Reconstitution proved also to be difficult for the peptide AOD-9604 of vendor Y. Addition of an extra mL of water and FA to 1% based on the total volume was required to dissolve all lyophilized powder. This effect was however not encountered for other samples of the same vendor or for the same peptide of different vendors.

3.1 Analysis of the API and API-related impurities

Analysis with LC-IT-MSⁿ of the native preparations and tryptic digests of AOD-9604, the hGH-RH-analogues and thymosine β 4 pointed out that all preparations indeed contained the claimed API, except for the polypeptide thymosine β 4. For this polypeptide, the label of the different preparations stated "thymosin β 4 (TB-500)", "TB-500" and "thymosin Beta 4" on the vials of respectively vendor X, Y and Z. TB-500 (Ac-LKKTETQ) is known in literature as the synthetic N-acetylated fragment (AA 17-23) of thymosine β 4^{19,45}. Interestingly, the N-acetylated 43 amino acid long native polypeptide was found and not the heptapeptide TB-500. Tryptic digests of all subjected peptides resulted for all preparations in sequence coverage of more than 95%.

Calculation of the variance in amount of API present between preparations of different vendors based on the absorbance at 214 nm of the API showed a low variation for the cosmetic peptide melanotan II (RSD of 2%). For the GHRP's RSDs of 10 to 24% were calculated while high RSDs were calculated for AOD-9604 (42%) and oxytocin (78%). These large variations in amount of API between the different vendors for oxytocin and AOD-9604 coincided with a low level of purity determined by UV at 214 nm. The calculated purity levels for these preparations were 74.8 and 71.7 % for oxytocin and 45.9 and 4.8% for AOD-9604. The GHRPs and melanotan II displayed high purity levels between 97.0 and

99.9% while the levels of the tetrapeptide epitalon varied between 80.1 to 94.6 % based on HILIC-UV analysis (table 3). For the peptides sermorelin, CJC-1295 and thymosin β 4 purity levels could not be calculated since no impurities could be detected via UV at a wavelength of 214 nm (or at 280 nm) above the reporting threshold of 0.1%. Table 3 shows all detected API-related impurities with a signal to noise ratio above 3.3 and a relative area of 0.1% compared to the AUC of the respective API at a UV wavelength of 214 nm and their suggested peptide sequence based on HRMS and MS² fragmentation patterns. Furthermore, supplemental table 2 contains all API-related impurities detected on LC-IT-MSⁿ with a relative area below the 0.1% threshold in the LC-UV chromatogram at 214 nm.

As can be derived from both tables, the three preparations of ipamorelin showed a reduced number of API-related impurities (C-terminal deamidation and +12 Da on the second amino acid) but with a relative area ten times higher than the two other GHRPs (GHRP-2 and GHRP-6). Both for GHRP-6 and GHRP-2, multiple related impurities were found and identified at MS-level, but not detected with UV. Although impurities were detected in the LC-UV chromatogram of GHRP-6 for vendor B and C detected peaks with UV could not directly be allocated to the corresponding impurity in the LC-MS chromatogram because of the differences in the obtained impurity profiles of the LC-UV and LC-MS chromatograms.

Interestingly, for melanotan II both the purity level, amount of API and impurity profile showed high resemblance in both the LC-UV and LC-MS chromatogram (MS and MS²-spectra) between both vendors (Figure 1). The cyclic structure of melanotan II resulted in the difficult identification of these related impurities on MS² fragmentation patterns. Furthermore, in both melanotan II preparations, most of the related impurities (same m/z and MS² fragmentation pattern) and the API itself were detected two times at different retention times in the LC-MS chromatogram. A possible explanation for this observation is the presence of diastereoisomers due to the presence of both D and L-amino acids.

For epitalon, HILIC chromatography was employed to separate the related impurities and mannitol from the API. For all three preparations only one related impurity peak, namely aspartimide or succinimide formation, was detected at levels from 5% to almost 20%. Contrary, to the relatively high level of purity for melanotan II and the GHRPs, relatively high amounts of related impurities were detected for the tetrapeptide epitalon and the intracyclic cystine bridge containing peptides AOD-9604 and oxytocin. Oxytocin of vendor Y contained multiple deamidations (see Figure 2) while the reduction of the cystine bridge (7-14) was the most important peptide in all preparations of AOD-9604. For oxytocin the preparation of vendor X was found to contain high amounts of dimers and even trimers while multiple deamidations in the preparation of vendor Y were the most significant related impurities. Both related impurities (deamidations and dimers) were found in the two preparations at MS-level. In preparation X, related impurities with the same molecular mass of oxytocin dimers were detected at two different retention times in the chromatogram. A possible explanation for this is the sense or antisense alignment of the two oxytocin molecules as previously described in literature^{46,47}. Moreover, a related impurity with a molecular weight consistent with a complex of three oxytocin equivalents covalently linked via three cystine bridges (MW= 3019.3176 Da, Δ = -2.70 ppm) was found to co-elute with the last oxytocin dimer. Dimers were also seen for preparation Y and Z of AOD-9604 albeit via only one cystine bridge compared to oxytocin. Also here, dimers were seen at different retention times in the LC-MS chromatograms for both preparations as a result of the different alignments. For the preparation of vendor Y also deletions of respectively Ile(4) and Gln(6) were detected and allocated to the exact position via tryptic digests.

Although no related impurities were detected for sermorelin, CJC-1295 and thymosine β 4 on the LC-UV chromatogram, analysis of these preparations of the native peptide and tryptic digest via LC-MSⁿ resulted in the identification of multiple deamidations, insertions/deletions

of amino acids, oxidations of methionine and acetylations. It was however not possible due to the low resolution of the ion trap MS to allocate deamidations of Asn and/or C-term detected via tryptic digests in both sermorelin preparations. As previously mentioned full length thymosin β 4 was found in all three preparations while for two preparations (X and Y) the label claimed the presence of the synthetic peptide TB-500, the actin sequestering active site of thymosin β 4. Also here, deamidation of Asn, acetylation of Lys and oxidations of Met and the deletion of Gln were identified via tryptic digests.

Furthermore, contaminations with another API (AOD-9604) were identified via LC-IT-MS for GHRP-2 of vendor Y and sermorelin of vendor X but not detected in the respective LC-UV chromatograms.

3.2 Analysis of small molecule contaminants

All samples were screened with GC-MS and LC-MS² for the presence of excipients, synthesis related products (small molecules), other drugs (e.g. testosterone) and unrelated chemical contaminations. As described in section 2.5.1 and 2.6.1 an extraction with methanol was performed prior to analysis with GC-MS and LC-MS². During the extraction large pellet formations were seen for 16 of the 27 samples. Analysis of the supernatant of samples with pellet formation with GC-MS displayed a large peak at 13.7 min which could be attributed to sorbitol/mannitol according to the NIST-database (Figure 3). Other peaks at 6.85 (1,4-dianhydromannitol), 7.5 min (possibly isosorbide) and a cluster of peaks between minute 9 and 10 MS-spectra indicated the presence of a degradation products of a saccharide (possibly a polyol) in the respective preparations with pellet (Figure 3). Additionally in these samples, a complementarily peak appeared on the UHPLC-IT-MS² chromatograms after 1.3 min with a m/z value of 418.1 Da in positive mode and 416.1 in negative mode. Injections of analytical grade sorbitol and mannitol on the LC-MS² and GC-MS confirmed the presence of mannitol

in these samples based on the retention time and respective MS and MS² spectra. Analysis of the solubilized pellet via LC-MS² also demonstrated the presence of high amounts of mannitol. In samples without a visual pellet, no mannitol was detected in either the GC-MS or respective LC-MS chromatograms.

In the GC-MS chromatograms (Figure 3), all observed peaks could be either attributed to the presence of mannitol or as chemical derivatives of the respective API by comparison with their respective analytical standards. Furthermore, via LC-MS², the distinctive MS and MS²-patterns of poly ethylene glycol (PEG), triton-X and an amidated fatty acids were found in multiple preparations at trace level. No other contaminations were detected (small molecule API's such as testosterone, scavengers or protective groups used during SPPS, ...).

3.3 Screening for elemental impurities

The methods for elemental analysis were suitable as trueness of the spike ranged 81-109% with the exception of the analysis of vanadium (trueness = 130%). It was not possible to remove all interference on V analysis with the current method. Trueness of the CRM (Tomato leaves NIST 1573a), a more complex matrix compared to the expected matrix in falsified peptide drugs, ranged between 88 and 98%.

One of the first observations, as can be seen in Table 2, when weighing the different samples was the variety in mass of the total content of the different peptide preparations. In addition, the low amount of material for the GH-analogues and GHRPs resulted for some samples in LOQs above the concentration limits set by the ICH for the respective elements even when pooling two vials of the respective sample. The most cumbersome observation was the exceedance for 7 samples of the limit for the class 1 elements arsenic (six samples) and lead (one sample). For arsenic, six samples were found to exceed the limit (1500 ppb) ranging from 1660 ppb to 12 890 ppb, more than ten times the allowed concentration. Subsequent

speciation analysis of a second batch of samples via HPLC-ICP-MS confirmed the elevated arsenic concentrations in these peptide drugs and revealed that all arsenic was present as inorganic arsenic (data not shown). Other exceedances of the established limits were found for copper (one sample), silver and barium. For all other elements relevant to risk assessment (cadmium, cobalt, lithium and antimony) no exceedances were measured while for mercury and nickel not enough material was available to exclude exceedances of the respective ICH limits. In none of the samples traces of metal catalysts from the platinum group were detected.

3.4 Analysis of residual solvents

Traces (ppb level) of at least one residual solvent were detected in every sample. More specifically, six residual solvents (ethanol, ethyl ether, dichloromethane, tetrahydrofuran, acetonitrile and toluene), one anti-oxidant (butylhydroxytoluene) and hexanal were detected and identified with the NIST database and afterwards corroborated by comparison with an analytical standard. No class 1 residual solvents were detected in any of the samples. Quantification was performed for all detected chemical products with the exception of hexanal. The alkyl aldehyde was already degraded by the time a certified reference standard for quantification purposes was available. Validation criteria for the calibration curves are displayed in Table 3. All maximum back calculated concentrations were well below the thresholds set by the ICH (see Table 4). Tetrahydrofuran (THF) was found in all samples within a range of 20 to 900 ppb. No trends or correlations between residual solvent content or concentrations and the respective peptides or vendors could be established.

4. Discussion

Ten of the most frequently encountered falsified peptide drugs on the Belgian market were analysed for the API, API-related impurities, small molecule contaminants, residual solvents and elemental impurities. The investigated peptides were acquired from three different suspected illegal internet pharmacies which sell their peptides under the cover of research chemicals. Although most of these peptides are used for doping and cosmetic reasons, comments on internet forums also mention the administration of GHRPs, hGH-RH-analogues, epitalon and thymosin β 4 to treat (chronic) joint and/or muscle injuries. Often users describe the origin, use and effect of their administrations on internet forums and warn each other of false preparations or preparations with acute side effects and low functionality. Consequently, the selection of the three most popular (and reliable) web-based vendors was based on their popularity on internet forums.

The downside of this selection procedure however was the inherent bias induced since the selected sample set may contain more samples with a relatively high quality compared to the average preparations available on the internet. Other imperfections are that not all quality aspects were analysed (e.g. counterion, correct configuration) and the use of different vials for each analysis due to different sample preparation techniques. For the determination of elemental impurities however the pooling different vials from the same sample was required to acquire sufficient amount of sample with the innate risk of levelling-out exceedances.

Nonetheless this study provides a general overview of potential health risks based on the analysis of critical quality points. Analysis of the API by means of LC-IT-MS² resulted in the identification of full length thymosin β 4 in the preparation of all three vendors while two vendors stated the presence of TB-500 on the label. TB-500 is the N-acetyl derivative of one of the active sites (major actin binding site) of the thymosin β 4, existing of only 7 amino

acids⁴⁸. It stands to reason that the synthesis of the N-acetylated heptapeptide is less costly and complicated than the 43 amino acid long thymosin β 4. Nonetheless, in Germany also thymosin β 4 was found in samples distributed as 'TB-500'¹⁶. Other reports in literature however report that TB-500 was indeed identified in confiscated vials in Belgium and Germany^{19,45}. Moreover, both thymosin β 4 and TB-500 are not authorized for medicinal use and the latter has no track record of its efficacy and safety. Accordingly, these findings support other reports in literature which describe the presence of unauthorized APIs or other APIs than indicated⁷. Evidently, the administration of unauthorized peptide drugs or falsified preparations with other APIs than labelled may constitute serious (unknown) health risks. Furthermore, the administration of APIs with implicit health risks (hGH, growth factors, hGH secretagogues and hormones,...) without medical guidance and supervision may also result in adverse (longterm) clinical outcomes¹³. Other issues which may cause adverse side effects are the potential low solubility, as encountered for one of the preparations, and the noncompliance of these solutions with regard to the criteria for injectable drugs (pH, osmotic pressure,...). A striking example, which is in line with our findings, is the advice between users on forums to take anti-histamine drugs when injecting these preparations to reduce side effects of the injection, drug itself or possible inflammatory contaminants.

Although no formal quantification of the detected peptides was performed in this study, this topic was already treated in an earlier study performed by Vanhee *et al.* in 2015 demonstrating that the vast amount of these preparations are under-dosed⁶, analysis of the variance in amount of peptide between vendors resulted in large differences. Particularly for oxytocin and AOD-9604 the variability on UV-response at 214 nm was relatively high, respectively 42% and 78%. This high variability in amount of peptide is probably not exclusively because of the varying amount of peptide between vendors but also a result of the relatively high amount of API-related impurities. For example, the semi-quantification of the

related impurities resulted in a purity of 74.8 (preparation X) and 71.7% (preparation Y) for oxytocin and respectively 45.9, 4.5 and 4.5% for preparation X, Y and Z of AOD-9604.

In contrast to the low purity of the cysteine containing peptides oxytocin and AOD-9604, no related impurities were detected for the wound healing peptide thymosin β 4 and the hGH-RH-analogues (sermorelin and CJC-1295) at the standard wavelength of 214 nm for the analysis of related impurities and the aromatic wavelengths (254, 280 nm). Possible reasons for the absence of API-related impurities at UV level may be the lower absorption of these compounds compared to the GHRPs and the low amount of API in the respective preparations. The two peptides with the highest amount of related impurities (AOD-9604 and oxytocin) were found to have alterations related to the intracyclic cystine bond in every preparation. More specifically, reduction of the cystine bond and to lesser extent the formation of dimers were the major related impurities for AOD-9604 while for oxytocin dimers and in one preparation even trimers were detected. In addition, also deamidations were found in both preparations of oxytocin. Moreover, in preparation Y of oxytocin deamidations were the major related impurities. Both deamidations and alterations in the oxidative state of cysteine can be formed during synthesis of the peptide but also during storage. These alterations can be influenced by pH, redox potential of the solution, and temperature. Therefore, the handling and storage of these peptides is crucial for the functionality of these preparations^{46,47}. Also other related impurities such as oxidations of Met and Trp, acetylations, deaminations (loss of ammonia), succinimide formation (loss of water) and crosslinking which can be formed (spontaneously) under particular conditions (pH, T, partial pressure of oxygen/hydrogen) were detected in every preparation. Other types of related impurities which mainly arise during synthesis such as uncleaved protective groups, deletions or insertions of amino acids and (possibly for CJC-1295) truncations were also detected but at much lower levels. Only one preparation, GHRP-2 of vendor X, was

found to contain an impurity (insertion of Ala) with a relative area higher than 0.1% based on the LC-UV chromatogram. Many more insertions or deletions were however detected with mass spectrometry (below the 0.1% relative area at UV level). Although no severe cases of insertions, deletions, truncations or wrong sequences were detected in our sample set, reports of literature dealing with related impurities in medicinal peptides (for research purposes) demonstrated the presences of high amounts of alterations during synthesis of these peptides^{49,50}. Possible explanations for these different observations may be the inherent bias in our study due to the selection of the most reliable and popular internet vendors, the putative challenging synthesis as result of demanding sequences or configurations and the intrinsic susceptibility of the selected peptides towards alterations during storage and handling. Moreover, the detection of numerous oxidations of methionine and tryptophan in multiple preparations, the succimide formation with epitalon and the deamidation of asparagine in sermorelin are probably a result of the intrinsic susceptibility of the peptide to degradation. More specifically, the latter peptide has been redesigned into CJC-1295 by modifying the sequence of sermorelin at 4 different places to elongate the chemical stability and half-life. One of the modifications is the substitution of Asn at position 8 to Gln, which should be (in general) less prone to deamination than Asn⁵¹. Indeed, no deamidations were detected for all three preparations of CJC-1295 which could result from the higher stability of CJC-1295 compared to sermorelin. Naturally, not only the intrinsic peptide stability but also the content of these preparations such as excipients or possible contaminations and external factors may influence the stability (and hence possible degradation rate and products) of the above mentioned peptides.

Nonetheless, both synthesis artefacts or modifications due to storage or degradation may lead to changes in functionality. Even the change of one amino acid from the L to D-conformation can change the potency of the respective peptide when coupled to a receptor while insertions

or deletions of amino acids may even transform agonist /-antagonistic functionality. In even worse cases certain API-related impurities such as aggregates (dimerization) may induce toxic responses or immunological reactions^{37,52-55}. For instance, oxytocin loses (reversibly) almost all (1/500) of its biological functionality upon dimerization⁴⁷.

Next to the API and related impurities only mannitol was detected in significant amounts. Possible reasons for the presence of high amounts of mannitol are its role as cryoprotectant during freeze drying and as filler substance⁵⁶. Furthermore, only trace amounts of triton-X, PEG and an amidated fatty acid were detected by means of LC-MS and GC-MS in multiple preparations. Since the latter three compounds were only detected at very low levels, the identification was only based on MS² fragmentation patterns and not corroborated by injection of an analytical standard. Residual solvent analysis by HS-GC-MS established the presence of six residual solvents (DCM, THF, ethanol, ethyl ether, toluene and acetonitrile) commonly used during SPPS at levels well below the respective toxicity limits. The presence of BHT can be explained by its role as additive in solvents as stabilizer due to its anti-oxidative properties. Finally, hexanal, also found in food as a degradation product of the β -oxidation of ω -6 fatty acids such as linoleic acid (18:2, n-6) has no known role in peptide synthesis. In general, the low amount and concentrations of residual solvents and chemicals used during synthesis can be explained by the efficient washing and filtering step during SPPS while mannitol is probably added after the purification of the peptide for the abovementioned reasons.

The presence of class 1 elemental impurities (As, Pb) however is most likely a result of contaminations rather than their use as catalysts during peptide synthesis as most of the detected elements are generally not known for their usage as catalysts (Zn, Au and platinum group elements) in the synthesis of peptides. Leaching from equipment (lab equipment, glass material, reactors, containers,...) or the usage of low quality reagents and solvents (including

water) are probable causes. The high amounts of inorganic arsenic in these preparations raises questions regarding the origin of the contamination(s). Known likely sources of arsenic contaminations in these samples are groundwater and glass^{57,58}

Both As and Pb are class 1 elemental impurities, meaning human toxicants with no use in the manufacture of pharmaceuticals. Although both elements are known as human carcinogens, their toxicity depends on their oxidation state and form. Lead has limited acute toxicity, but accumulates in soft tissues ($\pm 10\%$; brain, spleen, kidneys, liver and bonemarrow) and hard tissues (90%; bones and teeth). Chronic exposure to lead causes adverse effects on the central nervous system, hematopoietic system, the immune system, the renal system and the reproductive system. Untreated lead poisoning has been known to develop into encephalopathy, lethargy, delirium, convulsions, coma and even death^{59,60}.

The metalloid As, in the past a popular criminal toxin, has been linked in numerous literature reports to be carcinogenic (however not mutagenic) and has adverse effects on the skin, respiratory tract, central nervous system, cardiovascular system, liver and immune system (humoral and cellular response). The toxicity of As depends primarily on the excretion, metabolism (phase I and phase II biotransformation reactions) and the affected tissue(s). In literature, inorganic forms are regarded as the most toxic forms with the trivalent arsenite (As^{III}) has been found to be more toxic than arsenate (As^{V}). Nonetheless, certain As containing compounds (GSAO, Darinaparsin and Trisenox) are currently being used as medicine against leukemia, lymphomas and solid tumors for their anti-carcinogenic properties (by generation of ROS, immunosuppressive effects, anti-angiogenesis)⁶¹⁻⁶⁴.

Conclusion

In this study the ten most frequently encountered falsified peptides that were confiscated in seizures of Belgian customs and regulatory agencies between 2009-2017 were analysed for

the presence of multiple chemical impurities potentially present in these type of preparations. According to internet forums these preparations are used to heal muscle or joint injuries, as cosmetic or most importantly for doping reasons. Most of the preparations are however not authorized for their usage as parenteral medicine or are preclinical drugs for which no track record of their safety and efficacy has been established.

Moreover, our analysis demonstrated the presence of toxic class one elemental impurities (inorganic As and Pb) next to the high variations in content and high amounts of API-related impurities in these samples and thus clearly illustrates the health risks users/patients face when resorting to falsified peptide drugs. Both Pb and inorganic As are known (genotoxic) elemental impurities which in acute intoxications or prolonged exposure may lead to adverse health effects on multiple vital organs and in some cases also result in lethal outcomes. The presence of concentrations up to ten times the known toxicity limits for parenteral drugs raises questions about the quality of chemicals used and the purification process.

In addition, the low purity of certain compounds and high variations in amount of API makes it difficult to accurately administer biologically relevant doses and can easily lead to underdoses and overdoses. Furthermore, the presence of certain related impurities such as deamidations, insertion and deletion of amino acids and the formation of aggregates may lead to loss of functionality and even toxic effects. Most of the detected related impurities in these preparations (oxidations, deamidations, dimerformations,...) could also be induced during storage or handling under certain conditions. Hence, demonstrating that next to the analysis of the API and synthesis artefacts also degradation products (specifically for peptides which were not subjected to preclinical or clinical trials) may not be neglected in the risk evaluation of falsified peptide drugs.

Taken together, the results of this study clearly demonstrate and confirm the suspicions that these products do not meet the required safety regulations regarding the purity of the API and chemical impurities in these preparations. Consequently, the (sustained) administration of these preparations may result in adverse acute and chronic health effects.

Acknowledgements

The authors would like to thank Koen De Cremer, Sophia Barhdadi and Angelique Kamugisha for their support during the experimental work and writing of the article.

Conflict of interest

The authors state that there is no conflict of interest.

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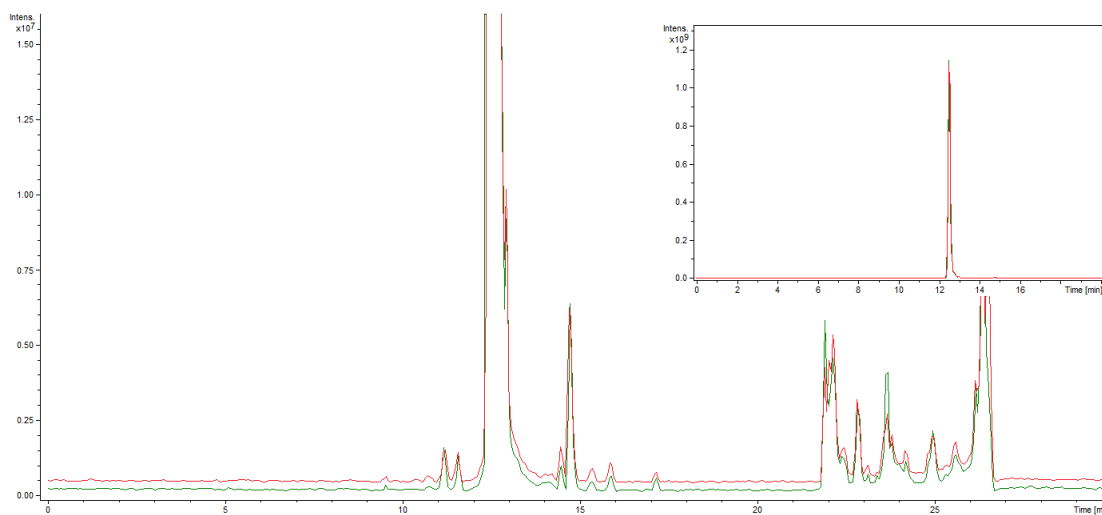


Figure 1: Total ion chromatogram (TIC) of Melanotan II from vendor X (red line) and vendor Y (green line), obtained by means of UHPLC-MSⁿ, showing the similarity in intensity, and related impurities.

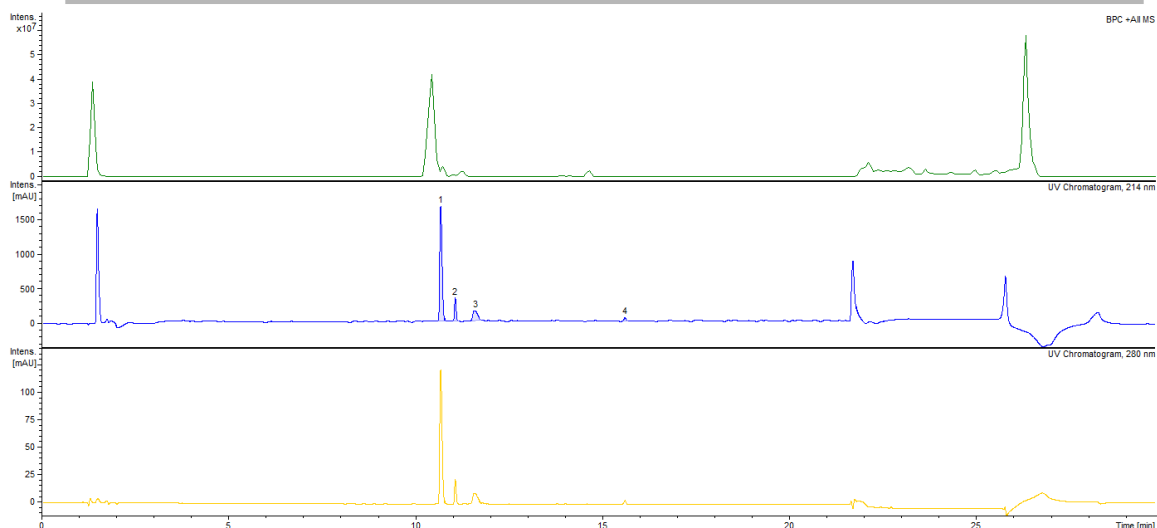


Figure 2: Total ion chromatograms obtained via UHPLC-MSn (top chromatogram) and UHPLC-UV chromatograms at 214 (middle) and 280 nm (bottom) of Oxytocin of vendor X showing on UV chromatogram at 214 nm; (1) API, (2) Deamidation, (3) 2*Deamidation, (4) Acetylation.

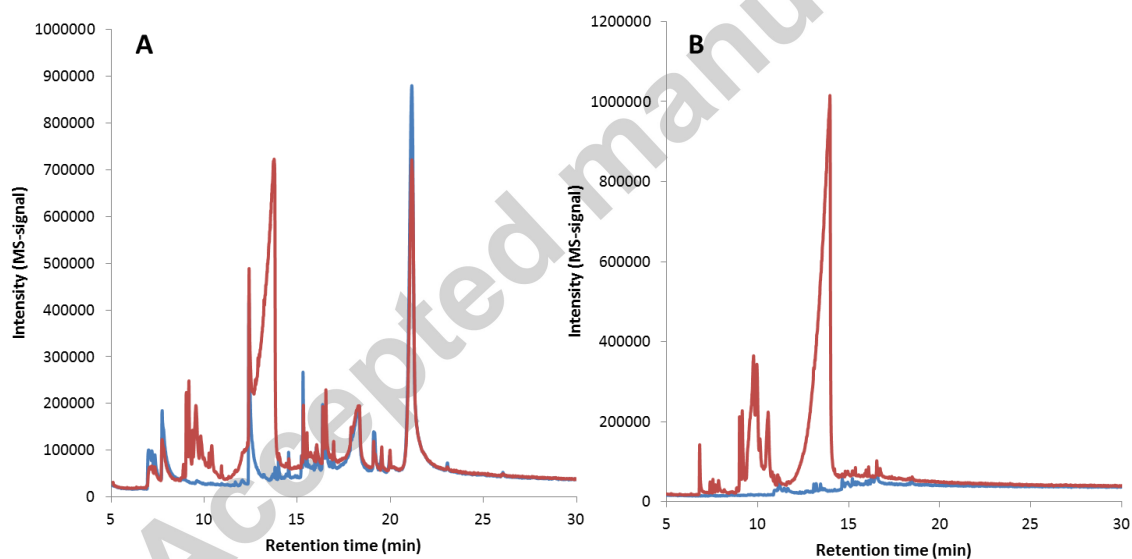


Figure 3 : Total ion chromatograms obtained by GC-MS of (A) GHRP-6 vendor Y (blue line) and vendor Z (red line) and (B) the hGH-rh analogue sermorelin of vendor X (blue line) and vendor Y (red line). Figure 3A shows high amounts of mannitol for the peptide preparation of vendor Z (peak at 13-14 min, and peak cluster at 10 min) and multiple peaks corresponding to GHRP-6 in both preparations. Figure 3B shows the high amounts of mannitol for the

preparation of vendor Y (red line) while no degradation products of sermorelin were detected for both preparations, possibly due to the low solubility in methanol.

Table 1: Polypeptides with molecular weight, sequence, most important functionality regarding illegal use and suspected internet based pharmacy (vendor)

Polypeptide	Monoisotopic MW (Da)	Sequence	Functionality	Vendor		
				X	Y	Z
AOD-9604	1813.9	YLRIVQ[CRSVEGSC]GF	Lipolytic	V	V	V
CJC-1295 w/o DAC	3365.9	Y(d-A)DAIFTQSYRKKVLAQLSARKLLQDILSR-NH ₂	hGH secretagogue	V	V	V
Epitalon	390.1	AEDG	Anti-aging	V	V	V
GHRP-2	817.4	(d-A)(d-2-Nal)AW(d-F)K-NH ₂	hGH secretagogue	V	V	V
GHRP-6	872.4	H(d-W)AW(d-F)K-NH ₂	hGH secretagogue	V	V	V
Ipamorelin	711.4	Aib-H-(d-2-Nal)-(d-F)K-NH ₂	hGH secretagogue	V	V	V
Melantani II	1023.5	Ac-Nle-cyclo[(β -D)-H(d-F)RW-(ϵ -K)]-NH ₂	Cosmetic	V	V	N A
Oxytocin	1006.4	[CYIQNC]PLG-NH ₂	Neurohormone	V	V	N

Sermorelin	3355.8	YADAIFTNSYRKVLGQLSARKLLQDIM	SR-NH2	hGH	secretagogue	V	V														
Thymosin β	4960.5	Ac-SDKPDMAEIEKFDKSKLKKKTETQEKNP	LPSKETIEQEKGAGES	Wound healing ^(*)																	

V: present, NA: Not Available, Nal: naphthaline, Aib: amino butyric acid, Pyr: pyroglutamic acid, Ac: acetyl, Nle: Norleucin, []: Intramolecular disulfide bridge, cylco[]: covalent intramolecular bridge

(*) most popular functionality

Table 2: Measured concentrations for all elements described in ICH guideline Q3D with their respective concentration limit for parental administration for drugs set by the ICH (Q3D guideline). Red marked values are measured concentrations above the set threshold, concentrations below the LOQ are presented as “< respective LOQ”. Elements with grey marked columns are used in risk assessment regarding public health. For each samples two vials were pooled, while only one vial was used if the amount is followed by an asterisks. The claimed amount takes into regard the number of vials used and the amount designated on the label of the respective vial(s).

Vendor	Claimed amount of API (mg)	Amount present in vials (mg)	Class 1 (ppb)							Class 2 (ppb)							Class 3 (ppb)							
			As	Cd	Hg	Pb	Co	Ni	Tl	Au	Pd	Ir	Os	Rh	Ru	Se	Ag	Pt	Li	Sb	Ba	Mo	Cu	Sn
X	4	14.3	78	<	<	35	1	<	<	<	<	<	<	<	<	<	<	<	<	74	13	<	<	<
			5	3	5	4	1	68	6	17	2	4	3	5	3	2	5	31	54	10	52	23	94	1
Y	4	20.1	89	<	<	25	4	1	48	4	12	2	3	<	<	<	<	<	<	30	17	<	<	<
			1	5	1	3	0	9	5	6	0	1	3	2	4	1	4	22	38	05	8	18	83	33
Z	4	19.5	26	<	<	25	4	1	50	4	12	2	3	<	<	<	<	<	<	37	76	19	86	35
			8	6	9	4	0	5	7	9	1	4	2	4	9	1	4	3	1	31	61	40	03	<
X	10	3.7	<	<	<	1	<	<	<	<	<	<	<	<	<	<	<	<	<	30	<	<	<	<
			82	1	13	7	5	26	2	68	1	7	1	1	2	13	7	2	47	21	80	19	89	39

			3	63	1	5	58	4	1	0	7	2	0	63	0	12	8	8	53	03
			6		8			5		9										
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	10	5.6	28	90	1	3	17	1	45	7	1	1	1	90	1	81	13	96	42	69
			89	0	3	6	56	2	0	2	5	7	8	4	5	4	1	96	8	5
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Z	10	6.3	78	80	1	3	15	1	40	6	1	1	1	80	6	11	12	93	68	25
			93	0	6	2	61	4	0	4	4	0	4	2	1	98	41	4	4	5
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
X	10	7.1	31	71	4	2	13	1	35	5	4	9	6	1	71	4	11	10	<6	29
			0	0	4	8	85	8	5	7	4	2	1	0	0	26	01	2	2	6
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	10	5.4	53	9	1	3	18	1	46	8	1	1	1	93	1	50	14	28	<8	38
			1	3	4	5	21	6	7	4	5	2	8	4	5	65	48	9	9	1
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Z	10	66(**)	16	8	1	3	14	1	38	6	1	1	1	77	1	18	11	49	<5	25
			8	77	3	9	9	4	1	1	0	1	1	1	1	5	9	40	1	1
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
X	20	14.5	74	3	5	4	93	6	17	2	2	4	3	5	34	2	53	92	<	11
			3	5	8	9	3	3	4	8	2	5	3	5	8	2	69	9	9	24
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	20	11.8	56	4	42	7	1	83	7	21	6	5	4	6	42	2	91	66	<	12
			2	3	7	3	7	3	7	4	7	2	6	4	7	2	8	2	2	27
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
X	10	46.6*	16	1	10	3	4	21	2	54	9	1	4	1	10	1	29	16	12	32
			60	1	8	1	1	0	0	0	1	4	1	2	8	1	8	8	5	92
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
X	10	33*	26	1	15	2	6	29	2	76	2	1	0	2	15	1	23	48	12	54
			5	3	3	2	8	8	8	2	1	1	0	1	3	5	7	1	3	12
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	10	43*	52	1	11	2	7	22	2	59	9	1	5	1	11	1	10	18	13	27
			2	7	2	9	1	9	1	1	5	1	2	1	2	6	2	7	5	7
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	10	41.5*	33	1	12	9	5	23	2	61	0	1	6	1	12	1	10	18	63	13
			2	2	9	5	7	2	2	0	1	6	1	2	2	9	8	4	4	13
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Z	10	64.3*	54	8	79	3	3	15	1	39	6	1	0	1	1	79	1	12	9	<4
			8	3	3	3	4	3	4	6	1	0	1	1	1	1	9	2	2	65
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Z	10	53.3*	51	9	95	8	4	18	1	47	8	1	2	1	1	95	1	14	66	<4
			9	8	4	5	7	5	7	8	1	2	1	1	1	1	1	7	6	3
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
X	4	20	25	2	25	4	1	49	4	12	2	1	3	2	4	25	1	22	39	19
			1	5	2	3	0	2	5	6	0	1	3	2	2	1	4	7	1	92
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	4	35.8	27	1	14	0	6	27	2	71	1	1	1	1	14	1	21	40	48	39
			33	4	1	0	6	5	5	1	1	8	1	2	1	52	9	9	0	17
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
X	4	56.1	49	9	90	8	4	17	6	45	7	1	2	1	1	90	1	14	02	<
			9	90	8	4	6	6	6	7	1	2	1	1	1	1	3	0	1	26
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	2*	27.8*	11	1	18	3	7	35	3	91	5	1	4	2	3	18	2	16	28	<
			38	8	2	1	7	4	3	5	1	4	2	3	2	2	3	3	1	5
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	2*	42.2*	19	1	12	2	5	23	2	60	0	1	6	1	12	0	10	18	92	16
			0	2	0	0	5	3	2	0	0	1	6	1	0	1	8	5	5	6
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Z	2*	27*	85	1	18	3	0	81	3	93	5	1	4	2	3	18	1	29	15	35
			9	7	3	0	3	3	4	5	1	1	4	2	3	7	3	0	0	91
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Z	2*	27.2*	59	1	18	3	9	81	3	93	5	1	4	2	3	18	1	28	75	23
			9	6	2	9	9	9	3	5	1	4	2	3	6	1	7	8	7	65
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	4	9.3	12	5	54	9	2	10	9	27	4	3	3	1	5	8	54	48	84	27
			1	4	2	2	2	58	8	1	3	3	1	5	8	2	2	3	8	58
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Z	4	8.7	74	5	58	9	2	14	1	29	4	3	5	5	9	58	3	52	89	78
			18	8	0	9	3	71	0	0	6	3	5	5	9	0	3	2	9	76
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
X	4	2.9	12	1	17	2	7	33	3	86	3	9	9	2	1	17	2	26	07	<
			89	4	39	6	0	91	3	9	9	6	6	6	6	39	5	6	95	28
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	2*	8.3*	74	6	60	0	2	11	0	30	4	3	9	5	9	60	3	54	94	<4
			7	1	8	3	4	85	0	4	9	9	9	3	9	8	3	7	2	7
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Y	2*	7.1*	48	7	71	2	2	13	2	35	5	4	9	6	1	71	4	63	11	14
			1	0	1	8	85	8	5	7	7	2	2	1	0	0	1	9	01	3
			<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
ICH Treshol d (ppb)			15	2	30	5	5	20	8	10	1	1	1	1	1	80	1	25	90	70
			00	0	0	0	0	00	0	0	0	0	0	0	0	00	0	00	00	00

(*) only one vial used

(**) two vials used, one vial had content of 4.4 mg while second vial had a content of 61.6 mg.

Table 3: All detected API-related impurities detected via UHPLC-DAD at 214 nm, with a relative area of minimum 0.1% and a S/N ratio of minimum 3.3, with the corresponding monoisotopic weight (Da) and deviation (Da) with the best fitting alteration based on HRMS and Ion trap MS² data. Furthermore the variation in amount of API between vendors and towards the mean amount of API are given for each peptides based on UHPLC-DAD data recorded at a wavelength of 214 nm. The table only contains peptides for which API-related impurities could be detected via UV at 214 nm. NYI: not yet identified, (*) co-eluting

Peptide	Vendor	$\frac{AUC_{AP}}{\langle AUC \rangle}$	RSD (%)	Purity (%)	MW (Da)	Rel Area (%)	Δ (Da)	Identification
	X	123%	24%	97.7	712.4	2.3%	1	C-terminal Deamidation
Ipamorelin	Y	102%		98.3	723.4	1.7%	12	NYI, +12 Da on His (2)
	Z	75%		98.6	723.4	1.4%	12	NYI, +12 Da on His (2)
GHRP-2	X	94%	11%	99.6	873.5	0.1%	56	Tert-Butylation on Lys or amidated C-term
					955.5	0.1%	138	NYI

	Y	93%		99.	873.5	0.1%	56	Tert butyl group on Lys or amide C-term
				4				
					955.5	0.1%	138	NYI
					955.5	0.1%	138	NYI
					859.5	0.1%	42	Acetylation N-term
	Z	113%		99.	821.5	0.1%	4	Oxidation of Trp to Kynurenine
				9				
	X	107%	9%	99.	914.5	0.1%	42	Acetylation of N-terminus
				6				
GHR P-6	Y	103%		98.				No matching peak in MS- spectrum
				2				
	Z	90%		97.				No matching peak in MS- spectrum
				0				
	X	98%	2%	98.	1083.	0.2%	60	NYI
				5	6			
					1083.	0.1%	60	NYI
				6				
					1023.	0.3%	0	NYI, Diastereoisomer?
Mela notan II				5				
					1024.	0.3%	1	Deamidation C-terminal
				5				
					1161.	0.1%	138	NYI
				6				
					1161.	0.4%	138	NYI
				6				

	Y	102%		98.	1083.	0.2%	60	NYI	
				5	6				
					1083.	0.1%	60	NYI	
					6				
					1023.	0.3%	0	NYI, Diastereoisomer?	
					5				
					1024.	0.3%	1	Deamidation C-terminal	
					5				
					1161.	0.1%	138	NYI	
					6				
					1161.	0.4%	138	NYI	
					6				
	X	70%	78%	74.	2012.	10.5	100	Dimerformation	
				8	9	%	6.4		
					2012.	13.0	100	Dimerformation	&
					9	%	6.4	Trimerformation(*)	
Oxyt					1048.	1.7%	42	Acetylation	
ocin					5				
	Y	130%		71.	1007.	22.2	1	Deamidation	
				7	4	%			
					1008.	6.1%	2	2* Deamidation	
					4				
Epital	X	89	22%	94.	373.1	5.4%	-18	Aspartimideformation	
on				6					
	Y	111		80.	373.1	19.9	-18	Aspartimideformation	

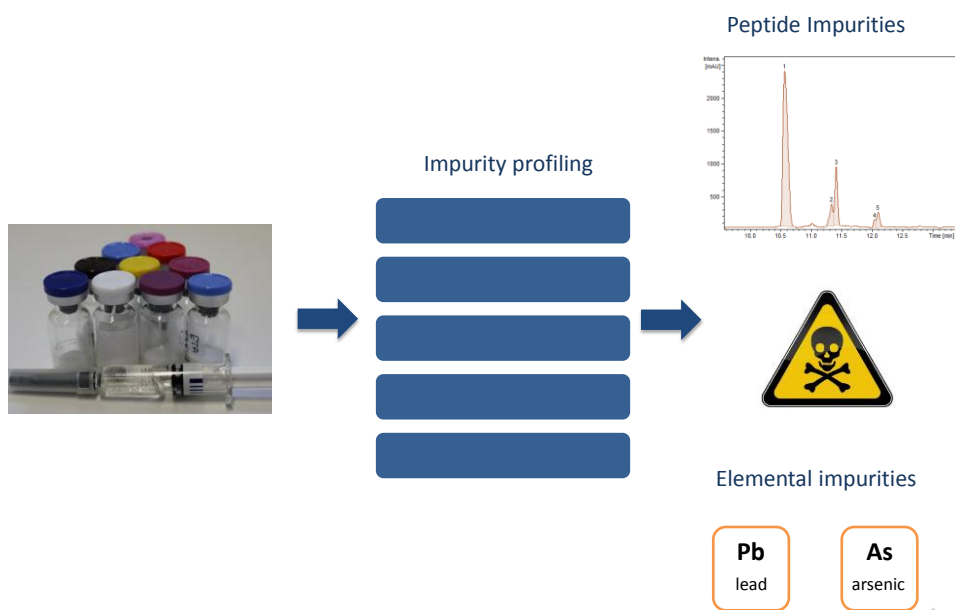
			1		%		
Z	102		89.	373.1	10.2	-18	Aspartimideformation
			8		%		
X	28%	42%	45.	1815.	54.1	2	Reduced cystine bridge (7-14)
			9	9	%		
Y	183%		4.8	1702.	3.3%	-	Loss of Isoleucine (4) +
			8			111	reduced cystine bridge (7-14)
			1815.	73.5	2		Reduced cystine bridge (7-14)
			9		%		
			1687.	3.9%	-		Loss of Glutamine (6) +
			8			126	reduced cystine bridge (7-14)
			3629.	7.3%	181		NYI; Dimerformation via one
AOD			8			6	cystine bridge
9604			NA	2.2%	NA		No matching peak in MS-
							spectrum
			3629.	5.0%	181		NYI; Dimerformation via one
			7			6	cystine bridge
Z	88%		4.8	1815.	78.7	2	Reduced cystine bridge (7-14)
			9		%		
			3629.	10.9	181		NYI; Dimerformation via one
			9		%	6	cystine bridge
			3629.	5.6%	181		NYI; Dimerformation via one
			9			6	cystine bridge

Table 4: All detected residuals solvents with the highest measured concentration (ppb) for every compound and the classification and toxicity limit according to the ICH Q3C guideline regarding residual solvents.

Compound	Highest Estimated Concentration (ppb)										Type of compound/ Residual solvent	Tox. Limit Ph. Eur. (ppb)
	Ipamorelin	GH RP-2	GH RP-6	Melanotan II	Oxytocin	AO D-9604	Epitalon	Sermoreline	CJC-1295	Thymosin β 4		
THF	892	765	884	477	496	326	321	264	263	248	Ph. Eur. Class 2	7.2·10 ⁵
DCM	---	21	---	97	---	---	---	---	---	---	Ph. Eur. Class 2	6.0·10 ⁵
Ethylether	---	---	266	---	---	---	---	---	---	---	Ph. Eur. Class 3	\geq 5.0·10 ⁶
Ethanol	---	---	6891	---	---	---	---	---	---	---	Ph. Eur. Class 3	\geq 5.0·10 ⁶
ACN	---	---	914	---	---	---	1240	---	2363	---	Ph. Eur. Class 2	4.1·10 ⁵
BHT	---	---	---	---	---	605	---	---	414	---	Anti-Oxidant	\geq 5.0·10 ⁶
Toluene	---	31	---	---	---	---	---	---	---	---	Ph. Eur. Class 2	8.9·10 ⁵

Highlights

- Overall amount of material present in the vials varied significantly between samples
- Different peptide samples contained different sequence dependent peptide impurities
- The lowest peptide purities were measured for cysteine containing peptides
- Multiple samples were contaminated with Pb and As above respective ICH toxicity limits
- Speciation of As demonstrated that all As was present in the more toxic inorganic form



Accepted manuscript