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Review Article

Immunoaffinity purification of peptide hormones prior to liquid chromatography–mass spectrometry in doping controls

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ABSTRACT

For most peptide hormones prohibited in elite sports the concentrations in plasma or urine are very low (pg/mL). Accordingly, hyphenated purification and enrichment steps prior to mass spectrometric detection are required to obtain sufficient doping control assays. Immunoaffinity purification in combination with nano-scale liquid chromatography coupled to high resolution/high accuracy mass spectrometry was found to have the potential of providing the necessary sensitivity and unambiguous specificity to produce reliable results. With the presented methodology 12 prohibited peptides (porcine insulin, Novolog, Apidra, Lantus DesB30–32 metabolite, Humalog and human insulin, Synacthen (synthetic ACTH analogue), luteinizing hormone-releasing hormone (LH-RH), growth hormone releasing hormone (GH-RH(1–29)) and CJC-1295 (GH-RH analogue), LongR^3-IGF-1 and IFG-1) were simultaneously purified from plasma/serum or urine. With limits of detection for each target compound ranging in the low pg/mL level (urine), the method enables the determination of urinary peptides at physiologically relevant concentrations. For each class of peptides an appropriate antibody and a respective internal standard was implemented ensuring robust analysis conditions.

Due to the fast and simple sample preparation procedure (~25 samples per day) and the fact that all materials are commercial available, the implementation of the methodology to laboratories from other analytical fields (forensics, pharmacokinetic sciences, etc.) is enabled.

1. Introduction

The World Anti-Doping Agency (WADA) has outlawed several potentially performance enhancing peptide hormones in elite sports [12]. Analytical approaches towards the detection of these prohibited peptides are primarily characterised by high sensitivity target analyses in biological fluids (urine or blood). The ultimate requirement of unambiguous result interpretation on the one hand and the low concentrations of the target substances on the other hand represent the major challenges to be overcome with state-of-the-art analytical methods [24]. Here, the combination of immunoaffinity (IA) purification with subsequent liquid chromatographic separation and mass spectrometric detection provides a powerful tool in doping controls as well as in related sciences (forensics, pharmacology, etc.) [2,5,9,14,16,23,25,26,28–33]. Successfully developed and implemented methods in this field are e.g. assays for the determination of intact (top-down) insulin, LH-RH (gonadorelin), Synacthen and IGF-1 in blood or urine [9,10,25,26,29–33]. Similar approaches are published for the prohibited proteins erythropoietin and human growth hormone [1,7,15,35]. The size of these target compounds commonly necessitates an additional enzymatic hydrolysis (bottom-up approach) to yield sufficient (proteotypical) peptides for the chromatographic separation and electrospray-assisted ionisation.

The biggest benefit of immunoaffinity-purified extracts is the absence of interfering matrix in the processed samples [8,22]. Considering the impact of the excess of interfering compounds in biological matrices on the ionisation under ESI conditions, the purity of the processed specimens is one of the key points in successful analytics [34]. Another crucial factor is the analyte concentration and the limited sample volume necessitating an isolation of the peptidic compounds from 1 mL of plasma or 5 mL of urine and reconstitution in 50 μL of injection solution. Under consideration of common recoveries between 30% and 100%, the resulting enrichment and purification is the item that eventually enables the successful detection employing modern mass analysers.

Abbreviations: LH-RH, luteinizing hormone-releasing hormone; GH-RH, growth hormone releasing hormone; CJC-1295, GH-RH analogue (tetrasubstitued); WADA, World Anti-Doping Agency; LOD, limit of detection; ISTD, internal standard; LC, liquid chromatography; MS, mass spectrometry; CID, collision induced dissociation.

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Table 1
Amino acid sequences of target peptides.

Peptide	Amino acid sequence
Porcine insulin	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTPKA
Novolog	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTDKT
Apidra	GIVEQCCTSICSLYQLENYCN – FVKQHLCGSHLVEALYLVCGERGFFYTPET
LantusMet	GIVEQCCTSICSLYQLENYCG – FVNQHLCGSHLVEALYLVCGERGFFYTPK
Humalog	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTKPT
Human insulin	GIVEQCCTSICSLYQLENYCN – FVNQHLCGSHLVEALYLVCGERGFFYTPKT
Synacthen	SYSMEHFRWGKPVGKKRRPVKVYP
LH-RH[*,**]	EHWSYGLRPG
GH-RH(1–29)[*]	YADAIFTNSYRKVLGQLSARKLLQDIMSR
CJC-1295[*]	YANAIFTQSYRKVLAQLSARKLLQDILSR
LongR^3-IGF-1	MFPAMPLSSLFVNGPRTLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA
IGF-1	GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA

[*] LH-RH, GH-RH(1-29) and CJC-1295: C-terminal amidated.
[**] LH-RH: N-terminus with dehydrated glutamic acid.

The methods discussed and presented within this manuscript are off-line IA purification methods that are realized on commercially available and commonly used liquid chromatography/mass spectrometry systems. A possible online-coupling was already reported for other analytical arenas, but a doping control method operating on a routine basis was not developed so far [4,13,17,19].

The ongoing technical and engineering developments for ultra-high performance liquid chromatography (UHPLC) and high resolution/high accuracy mass spectrometry (HRMS) devices in combination with down-scale processes into the nano-flow dimensions (nano-UHPLC coupled to nano-electrospray ionisation) during the last decades enable the detection of prohibited peptides after IA purification at low pg/mL (corresponding to low fmol/mL to amol/mL) concentrations. In addition to the fact that the determination of endogenous levels of insulin or IGF-1/2 is facilitated in blood and urine with these LODs, the detection window after an illicit application of synthetic peptide analogues is significantly prolonged [30,32].

The presented method enables the determination of 12 prohibited peptides comprising 6 insulins (porcine insulin, Apidra, Novolog, Lantus DesB30-32 metabolite, Humalog and human insulin), one synthetic ACTH analogue (Synacthen), one LH-releasing hormone (LH-RH), two growth hormone releasing hormones (GH-RH(1–29) and CJC-1295), and two IGF-peptides (LongR^3-IGF-1 and IGF-1). The amino acid sequences of the selected prohibited peptides are shown in Table 1.

2. Methods

2.1. Chemicals and reagents

Acetic acid (glacial), acetonitrile (analytical grade), sodium dihydrogenphosphate dihydrate (p.a.), disodium hydrogenphosphate dodecahydrate (p.a.), and sodium chloride (p.a.) were obtained from Merck (Darmstadt, Germany). The OASIS solid phase extraction cartridges were from Waters (Eschborn, Germany) and the paramagnetic secondary antibody-coated (anti-rabbit, anti-mouse IgG raised in sheep and protein A coupled) beads were from Invitrogen (Karlsruhe, Germany). The primary IGF-1 antibodies (polyclonal, host: rabbit), LH-RH antibodies (polyclonal, host: rabbit), GH-RH antibodies (polyclonal, host: rabbit), ACTH antibodies (polyclonal, host: rabbit) and insulin antibodies (monoclonal, host: mouse) were obtained from CER-Group Laboratories (Marloie, Belgium). All antibodies were pre-purified with protein A. For all dilution steps and preparation of aqueous solutions ultra pure water in MilliQ-quality was used. Insulin analogues Lispro (Humalog), Aspart (Novolog), Glulisine (Apidra) and Glargine (Lantus) were supplied by Eli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ), and Aventis (Kansas City, MO). Long-R^3-IGF-1, Porcine insulin and bovine insulin were from Sigma (Deisendorf, Germany). LH-RH reference substance was supplied as pharmaceutical formulation Kryptocur® by Sanofi-Aventis (Frankfurt, Germany). Synacthen Depot 1 mg was from Novartis Pharma (Bern, Switzerland) and the labelled analogue D_8-Synacthen (ISTD2) was obtained from BMFZ (Düsseldorf, Germany). CJC-1295 was obtained from Purepetides (San Diego, CA) and GH-RH(1–29) (Geref) was purchased from BMFZ (Düsseldorf, Germany). Des-pGlu-LH-RH (ISTD3) and Acetyl-(Tyr1,D-Arg2)-GRF(1–29) (ISTD4) were from Bachem (Bubendorf, Switzerland). Human IGF-1 and R^3-IGF-1 (ISTD5) were obtained from IBT Biosystems (Reutlingen, Germany).

Phosphate-buffered saline (PBS, pH 7.4) was prepared by dissolving 8 g of sodium chloride, 2.9 g of disodium hydrogenphosphate dodecahydrate, and 0.2 g of sodium dihydrogenphosphate dihydrate in 1 L of water.

Insulin glargine (Lantus) is not excreted intact into urine and, thus, the DesB30-32 truncated form (LantusMet) was produced by enzymatic hydrolysis (LysC) from Lantus reference compound. Details are described elsewhere [32,33].

2.2. Sample treatment

In contrast to doping control methods for small molecules the sample pre-treatment is a major concern. Due to the fact that peptidic target compounds are sensible against oxidation, enzymatic proteolysis and degradation, the storage of blood or urine samples in defined conditions is of utmost importance [30,31]. Freezing at −20 °C is an adequate way to preserve the specimens and avoid degradation. Considering the fact that cooling at 2–8 °C is the common condition for transport and storage of doping control samples, the analysis of sensitive peptides transferred or stored under cooled conditions only is ineffective and might lead to false negative results.

2.3. Internal standard (ISTD)

Five ISTDs were used to control the procedure for the different insulins (bovine insulin as ISTD1), Synacthen (D_8-Synacthen as ISTD2), LH-RH (Des-pGlu-LH-RH as ISTD3), GH-RH and analogues (Acetyl-(Tyr1,D-Arg2)-GRF(1–29) as ISTD4) and IGF peptides (R^3-IGF-1 as ISTD5). A mixture of these ISTD's were spiked with 500 pg/mL into each specimen (blood or urine) as the first sample preparation step.

Another benefit of adequately chosen ISTDs is provided by a distinct carrier effect on the corresponding target compound. That means that the ISTD, added to the sample in an approximately 10-fold higher amount than the expected target analyte's concentration, serves as a carrier for the measures and supports lowering