Forensic Science International 213 (2011) 35–41

Contents lists available at ScienceDirect

Forensic Science International

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

Doping control analysis of selected peptide hormones using LC–MS(/MS)☆

Mario Thevis *, Andreas Thomas, Wilhelm Schänzer

Institute of Biochemistry – Center for Preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany

ARTICLE INFO

Article history:
Received 29 April 2011
Received in revised form 14 June 2011
Accepted 14 June 2011
Available online 12 July 2011

Keywords:
Sport
Doping
Mass spectrometry
Peptide hormones
IGF-1

ABSTRACT

With the constantly increasing sensitivity and robustness of liquid chromatography–mass spectrometry-based instruments combined with enhanced reproducibility as well as mass accuracy and resolution, LC–MS(/MS) has become an integral part of sports drug testing programs particularly concerning the detection of peptide hormones. Although several of the relevant peptidic drugs such as insulins (Humalog LisPro, Novolog Aspart, etc.), growth hormone releasing peptides (GHRPs, e.g., GHRP-2, GHRP-6, Hexarelin, etc.), and insulin-like growth factors (e.g., IGF-1, IGF-2, long-R^3-IGF-1) are currently analyzed using dedicated top-down analytical procedures, i.e. employing specifically tailored sample preparation procedures followed by targeted LC–MS(/MS) measurements focusing on intact analytes, first approaches towards multi-analyte methods have been established. These allow the determination of the prohibited substances in blood and urine doping control specimens following therapeutic applications. In addition, the use of new complementary devices such as ion mobility analyzers, e.g., in hybrid mass spectrometers yielded promising data for the differentiation of isobaric insulins, which outlines the potential to further accelerate and multiplex doping control analytical assays to meet the continuously increasing demands of rapid and unambiguous test methods. Moreover, the potential of LC–MS/MS to target recombinant peptide hormones such as human growth hormone using bottom-up approaches has been demonstrated by targeting proteotypic peptides that unambiguously differentiate the recombinant molecule from the naturally occurring and endogenously produced analog.

1. Introduction

The detection of low molecular weight analytes in sports drug testing has been predominantly accomplished by means of various different chromatographic–(tandem) mass spectrometric techniques for several decades [1,2]. In contrast, the routine analysis of peptidic compounds by mass spectrometry has yet to be established in the doping control analytical arena, despite the fact that numerous methods were developed over the last 10 years that proved their fitness-for-purpose in (pilot) projects and case studies within as well as outside the sports drug testing territory [3–7]. The mass spectrometric equipment that has been employed in these studies included state-of-the-art instruments such as triple quadrupole (QqQ), ion trap (IT), quadrupole-linear ion trap (QqLIT), linear ion trap-orbitrap (LIT-orbitrap), and quadrupole-time-of-flight (QTOF) analyzers, interfaced to liquid chromatographs by electrospray or nanospray ion sources [2,8].

Among the peptide-based therapeutics and potential future drug candidates relevant for sports drug testing, synthetic and animal insulins, growth hormone releasing peptides (GHRPs), insulin-like growth factors, and human growth hormone (hGH) shall be highlighted in this mini-review concerning their traceability in blood and urine samples, the identification of urinary metabolites, as well as the characterization of artificial modifications in approved but also illicit products. Moreover, an outlook regarding analytical strategies possibly complementing, accelerating, and/or corroborating currently employed methods using ion mobility devices in hybrid quadrupole-time of flight mass spectrometers (QTOF-MS) is given.

2. Insulins

The use of insulins has been prohibited in elite sport since 1999, and street talk as well as several athletes' confessions rather than adverse analytical findings have defined the assumed breadth and dimensions of insulin abuse among sportsmen [9]. Especially the fact that insulin injections remained undetectable and below the analytical radar of doping control laboratories until 2005/2006 (when first detection methods were presented) [10–14] provided the 'safety' that a cheating athlete will not be caught when administering the antidiabetic drug. Among the presumed

☆ This paper is part of the special issue entitled: Fight Against Doping in 2011, Guest-edited by Neil Robinson (Managing Guest Editor), Martial Saugy, Patrice Mangin, Jean-Luc Veuthey, Serge Rudaz and Jiri Dvorak.

* Corresponding author. Tel.: +49 221 4982 7070; fax: +49 221 497 3236.
E-mail addresses: m.thevis@biochem.dshs-koeln.de, thevis@dshs-koeln.de (M. Thevis).

0379-0738/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.forsciint.2011.06.015

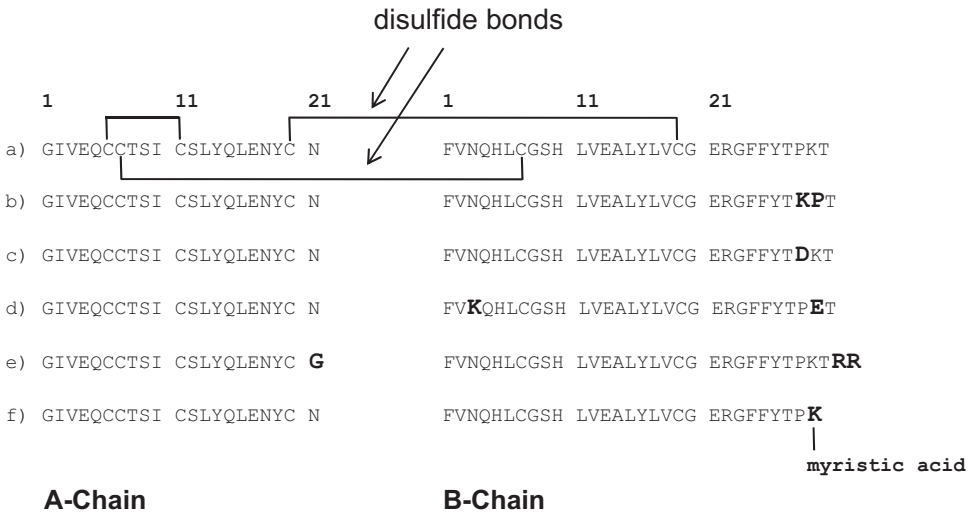

Fig. 1. Primary structures of human insulin (a) and its derivatives Humalog Lispro (b), Novolog Aspart (c), Glulisine Apidra (d), Lantus Glargine (e), and Levemir (f).

advantageous aspects, accelerated recovery (based on an increased incorporation of glucose into target tissues) and decelerated catabolism (due to its chalonic action concerning protein breakdown) in- as well as out-of-competition have supposedly been of particular importance [15].

The detection of synthetic insulin in human blood and urine samples for doping control purposes by means of mass spectrometry was firstly reported in 2005 and 2006 for rapid- and slow-acting synthetic insulins, which bear modified amino acid sequences compared to the naturally occurring version (Fig. 1) [10,11]. Utilizing the fact that either the molecular mass and/or the amino acid sequences differ between human insulin (Fig. 1a) and its modified analogs such as Humalog Lispro (Fig. 1b) or Glulisine Apidra (Fig. 1d) allowed for the combined extraction of insulin-like molecules by means of immunoaffinity purification followed by the separation and identification of target compounds by LC–MS(/MS). The required sensitivity to measure physiologically relevant concentrations of insulins in blood (fasting: 0.1–0.5 ng/mL, non-fasting: 0.2–3.2 ng/mL) [16,17] and urine (0.06–0.3 ng/mL) [18,19] was assured by nano-ultrahigh performance liquid chromatography (nanoUHPLC) and nanospray-tandem mass spectrometry, which in turn necessitated highly purified sample extracts accomplished by sophisticated sample preparation strategies [14]. In addition to the detection of the intact molecules of insulin and its derivatives, also metabolic products have been characterized from and detected in human urine [12]. A brief overview of the sample pretreatment is presented in Fig. 2. Commonly, 5 mL of urine are enriched with the internal standard (500 fmol of bovine

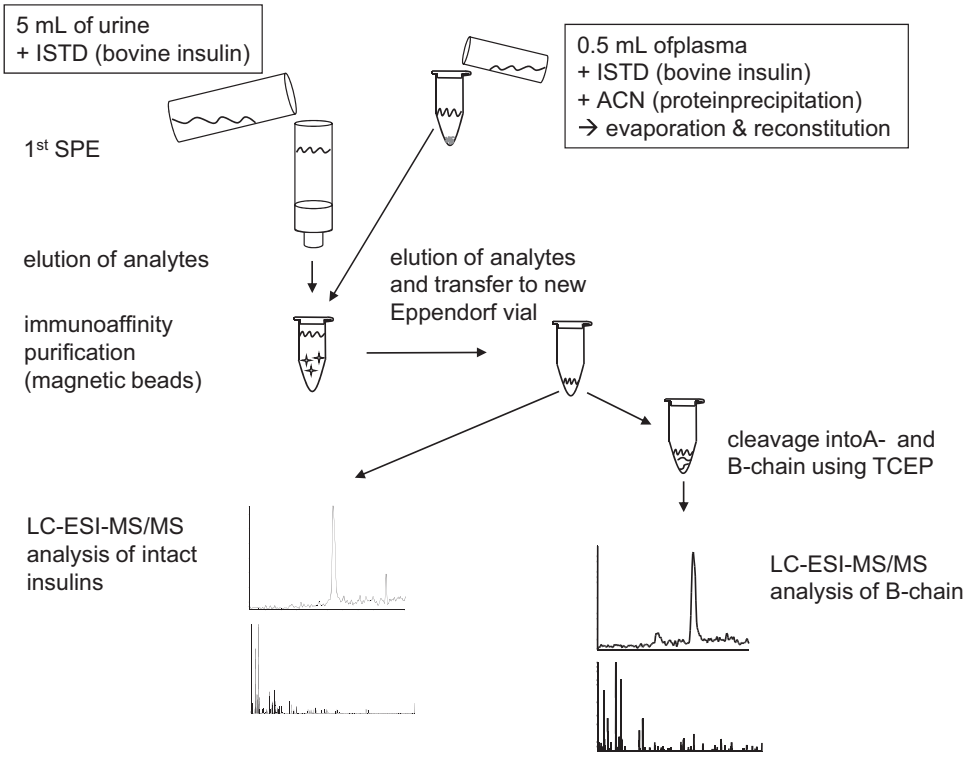

Fig. 2. Sample preparation strategy for the isolation and analysis of peptidic compounds from plasma/serum or urine.

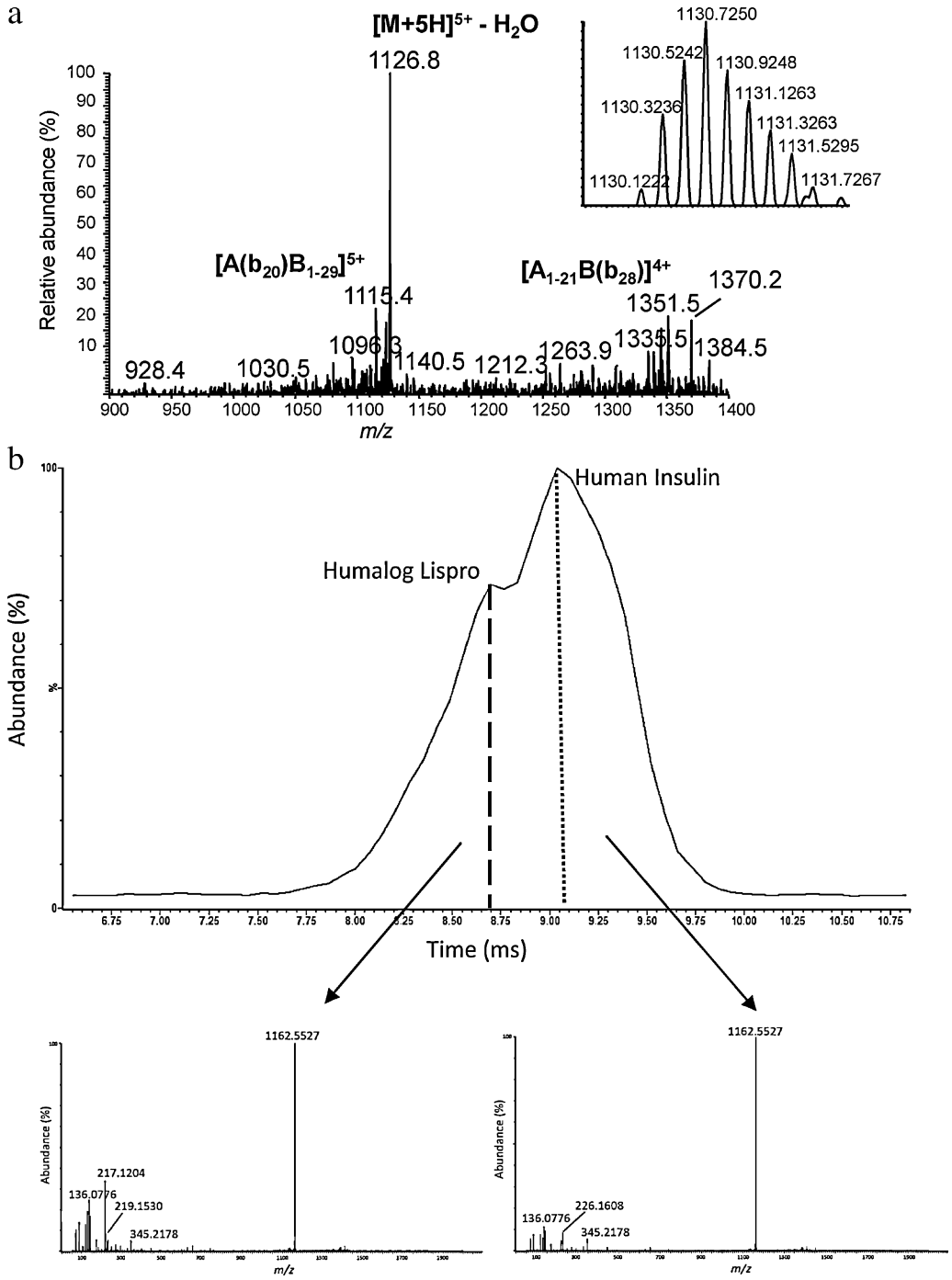

Fig. 3. (a) Product ion mass spectrum of the five-fold charged molecule $[M+5H]^{5+}$ at m/z 1130.7 (inset) of the DesB30-32 metabolite of Lantus Glargine as measured from a plasma specimen; (b) ion mobilogram (top) of the five-fold charged molecules $[M+5H]^{5+}$ of Humalog Lispro and human insulin at m/z 1162.5 and corresponding product ion mass spectra (bottom) obtained by time-aligned fragmentation of mobility-separated precursor ions. The diagnostic product ions at m/z 217 and 226 unambiguously characterize the B-chain C-termini of Humalog Lispro and human insulin, respectively.

insulin) and extracted on a C-18 mixed-mode adsorber resin. After elution with acetonitrile/water (80:20, v:v) the extract is concentrated to dryness, reconstituted in phosphate-buffered saline (PBS), and monoclonal anti-insulin antibodies are added followed by the addition of secondary antibody-coated magnetic beads. After incubation (2 h) and washing (PBS), the target analytes are released from the antibody–antigen complex by means of 2% acetic acid and transferred into an Eppendorf tube for LC–MS/MS analysis. In case of plasma or serum samples, the procedure includes a protein precipitation step followed by the reported immunoaffinity purification using magnetic nanoparticles. Also here, the captured analytes are liberated from the magnetic beads after washing and the pre-concentrated measurants are subjected to LC–MS/MS analysis [14]. The product ion mass spectrum resulting from an authentic plasma specimen is presented in Fig. 3a, demonstrating the identification of the Lantus Glargine (Fig. 1e) DesB30-32 metabolite (requiring a 35 min chromatographic run). The unambiguousness of the methodology has also been appreciated in forensics, and several cases of surreptitious insulin administration were proved, e.g., in an 8-week-old infant [6]. The long chromatographic run time and constantly increasing demand on higher throughput has required

further optimization, particularly concerning a faster LC–MS/MS analysis. In this regard, the utility of ion mobility combined with (tandem) mass spectrometry [20] was recently evaluated, especially supporting the separation of isobaric insulin molecules such as human insulin and its rapid acting derivative Humalog Lispro. Using a Waters Synapt G2 HDMS quadrupole time-of-flight/ion mobility spectrometer (QTOF/IMS, Manchester, UK) the five-fold charged molecules of human insulin and Humalog Lispro $[M+5H]^{5+}$ at m/z 1162.5 were separated by their respective drift times. Subsequent time-aligned fragmentation yielded the diagnostic product ions at m/z 226 and 217, corroborating the identification of human insulin (the less compact molecule with a longer drift time and a B-chain C-terminus generating the $y_3 - y_1$ ion at m/z 226) and Humalog Lispro (the more compact molecule with a shorter drift time and a B-chain C-terminus generating the y_2 ion at m/z 217), respectively as illustrated in Fig. 3b. Representing a pilot study at this stage, the potential of such a complementary dimension of ion separation is evident and might contribute to accelerated and more comprehensive sports drug testing methods in the future.

3. Growth hormone releasing peptides (GHRPs)

Growth hormone releasing peptides (so-called GHRPs) belong to a class of peptidic releasing hormones (Table 1) that significantly stimulate the secretion of human growth hormone (hGH) from the pituitary [21] and are prohibited according to the regulations of the WADA as "releasing factors" [22]. Besides their general ability to increase hGH and presumably provide an advantage due to the shortly elevated hGH plasma level, GHRPs reportedly possess the potential to mask a recent injection of recombinant hGH (rhGH) by counteracting the suppressive effect of the injected growth hormone on the natural production and secretion of hGH [23]. In administration studies with 100 μg (i.v.) of GHRP-2 (Pralmorelin, Table 1), the significant increase of serum hGH up to 191 ng/mL without resulting in an adverse analytical finding using commonly employed rhGH detection assays was reported. Since the mixture of growth hormone isoforms secreted from the pituitary upon Pralmorelin application comprised a natural composition, the immunoassay differentiating the predominant presence of the 22 kDa variant from other hGH isoforms was not appropriate to uncover the use of the releasing factor. In addition, further studies with rhGH injections (0.04 mg/kg) followed by a pralmorelin (100 μg i.v.) application 2 h later demonstrated the ability of the growth hormone releasing peptide to camouflage the rhGH administration. Consequently, the necessity to comprehensively probe for GHRPs as well as for growth hormone releasing hormones such as Sermorelin (GHRH 1–29, Table 1), which was suspected to be an integral part of doping practices among cyclists [24], has been recognized and methods based on LC–MS(/MS) analysis were established. One assay targeting the intact drugs in plasma was presented, which uses a protein precipitation and analysis of the supernatant employing a UHPLC-high resolution/high accuracy MS system [25]. In brief, 50 μL of plasma were fortified with a deuterated internal standard and mixed with 100 μL of acetonitrile. Following centrifugation to remove the precipitated proteins, the supernatant was analyzed by LC-ESI-high resolution/high accuracy orbitrap MS. Here, detection limits of approximately 5 ng/mL for GHRP-2 and hexarelin were accomplished. Complementary, procedures targeting GHRP-2 and its major metabolite (D-Ala-D-(β-naphthyl)-Ala-Ala-OH) in human urine after intravenous or oral application of GHRP-2 were published, exploiting the much higher frequency of urine sampling compared to blood testing for doping control purposes [26,27]. For urine specimen analysis, a total of 2 mL was spiked with 20 ng of the deuterated analogs to GHRP-2 and its main metabolite prior to weak cation exchange SPE. The isolated analytes were eluted, the eluate evaporated to dryness and the residue dissolved in 50 μL of LC buffer for subsequent LC-ESI-high resolution/high accuracy orbitrap MS. In order to provide adequate detection windows, sensitivity was of utmost importance and lower limits of detection of 0.2–1.0 ng/mL were achieved. An extracted ion chromatogram of a urine specimen collected 11 h after oral administration of

Table 1
Primary structures of peptides with growth hormone releasing activity.

Compound	Sequence
GHRP-1	Ala-His-D-βNal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-2	D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2
GHRP-4	D-Trp-Ala-Trp-D-Phe-NH_2
GHRP-5	Tyr-D-Trp-Ala-Trp-D-Phe-NH_2
GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2
Hexarelin	His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
Ipamorelin	Aib-His-D-2-Nal-D-Phe-Lys-NH_2
Alexamorelin	Ala-His-D-Mrp-Ala-Trp-D-Phe-Lys-NH_2
Sermorelin[a]	YADAIFTNSY RKVLGQLSAR KLLQDIMSR-NH_2

Non-standard abbreviations: Aib, aminoisobutyric acid; Nal, naphthylalanine; Mrp, 2-methyltryptophane.
[a] In one-letter-code.

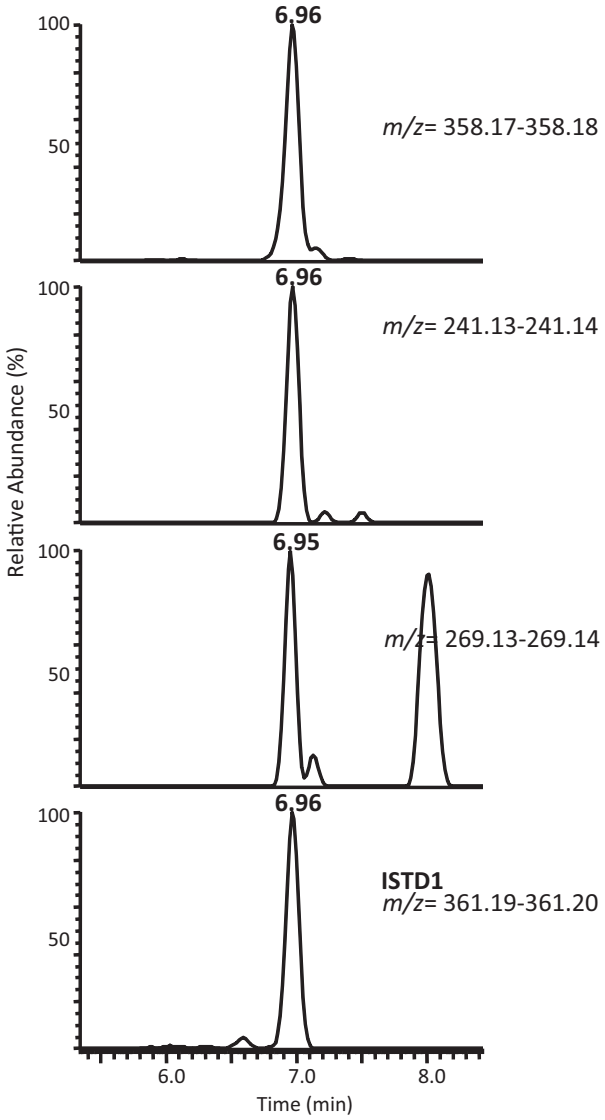

Fig. 4. Extracted ion chromatograms of a urine sample collected 11 h after oral administration of 10 mg of GHRP-2. The main metabolite is observed at 6.96 min.

10 mg of GHRP-2 is presented in Fig. 4, showing the diagnostic product ions of the main metabolite at 6.96 min and the internal standard (ISTD, $^{2}H_3$-D-Ala-D-(β-naphthyl)-Ala-Ala-OH). While this growth hormone releasing peptide has been studied concerning its renal elimination and metabolism for sports drug testing, comparable studies with other GHRPs are not yet available and, thus, target compounds for urine analysis still need to be defined. Hence, the currently most effective options to detect the misuse of as many GHRPs as possible are to focus on intact molecules in plasma or serum samples or to screen for *in silico* predicted metabolic products and comprehensive data acquisition (e.g., full scan high resolution/high accuracy mass spectrometry) since confiscations at custom controls have shown a considerable trafficking of GHRPs [28–30].

4. Insulin-like growth factors

The analysis of insulin-like growth factors (IGFs), in particular the determination of IGF-1 and its synthetic derivatives (Fig. 5), has become an essential aspect of sports drug testing. The importance of IGF-1 for doping control purposes predominantly arises from its mediating function with regard to several (especially anabolic) effects of human growth hormone (hGH) [9,31], some of which can be accomplished also by administration of IGF-1 itself. IGF-1 is available as therapeutic agent and, in addition, synthetic analogs to IGF-1 such as long-R^3-IGF-1 have been investigated as potential drug candidates but have not (yet) received clinical approval. Consequently, the quantitative analysis of IGF-1 has been desirable to accurately measure plasma (or serum) levels of this naturally occurring peptide hormone, which is an integral part of the so-called marker approach towards the detection of hGH (and potentially IGF-1) misuse by athletes [32]. Moreover, the qualitative analysis of modified IGF-1 (e.g., long-R^3-IGF-1) needs consideration in doping controls as underground literature [33] and seized compounds indicate a growing (black) market for illicitly produced and/or distributed substances related to IGF-1. Hence, several different approaches were reported to detect and quantify IGF-related peptides for sports drug testing purposes in plasma, serum, and urine using mass spectrometry.

Early procedures aiming the analysis of IGF-1 and its derivatives from blood samples employed immunoaffinity purification followed by LC–MS(/MS) measurements [34–36]. Due to the high affinity of IGF-1 to its binding proteins (IGFBPs), the liberation of IGF-1 from the non-covalent complex is an important sample preparation step, which is complicated by the fact that, following the release, an efficient binding to the anti-IGF-1 antibody is required. This issue was addressed by the careful selection and use of acidic extraction or addition of detergents prior to immunoaffinity isolation, and detection and quantitation limits for IGF-1 and its analogs by top-down analysis were achieved between 25 and 50 ng/mL. More recently, a bottom-up approach for serum samples employing protein precipitation and trypsin digestion followed by isotope-dilution mass spectrometry was presented [37]. Using the $^{13}C_6{}^{15}N_4$-labeled T1 peptide of IGF-1 as internal standard, IGF-1 was quantified from 30 μL serum aliquots with adequate sensitivity and good correlation compared to immunological methodologies. The short analytical run time (5 min) is of great advantage, while the required enzymatic hydrolysis proved to be the rate-limiting step which was not under control of the internal standard. Fully labeled IGF-1 would be the ideal internal standard compensating for issues potentially interfering at any sample preparation or analysis step, which would serve as reference in top-down as well as bottom-up methods [38].

Only one procedure was published so far targeting IGFs (IGF-1, IGF-2, long-R^3-IGF-1, Des1-3-IGF-1, etc.) in urine [7]. Employing a comparable sample preparation and analytical strategy as described for the insulin detection (Fig. 2), the analytes of interest were isolated from urine specimens (5 mL aliquots) by adding acid-ethanol (to break the IGFBP complex) followed by an ultrafiltration using a 30 kDa cut-off centrifugal device. Subsequently, the retentate was reconstituted and further purified by mixed-mode C-18 solid-phase extraction, and by means of dedicated antibodies and secondary antibody-coated magnetic nanoparticles the IGFs were retrieved for top-down LC–MS(/MS) measurement. Sufficient sensitivity (20–50 pg/mL) was accomplished by using nanoUHPLC, nanospray ionization, and tandem mass spectrometry, which also revealed the urinary degradation product of long-R^3-IGF-1, namely Des1-10-long-R^3-IGF-1, as the

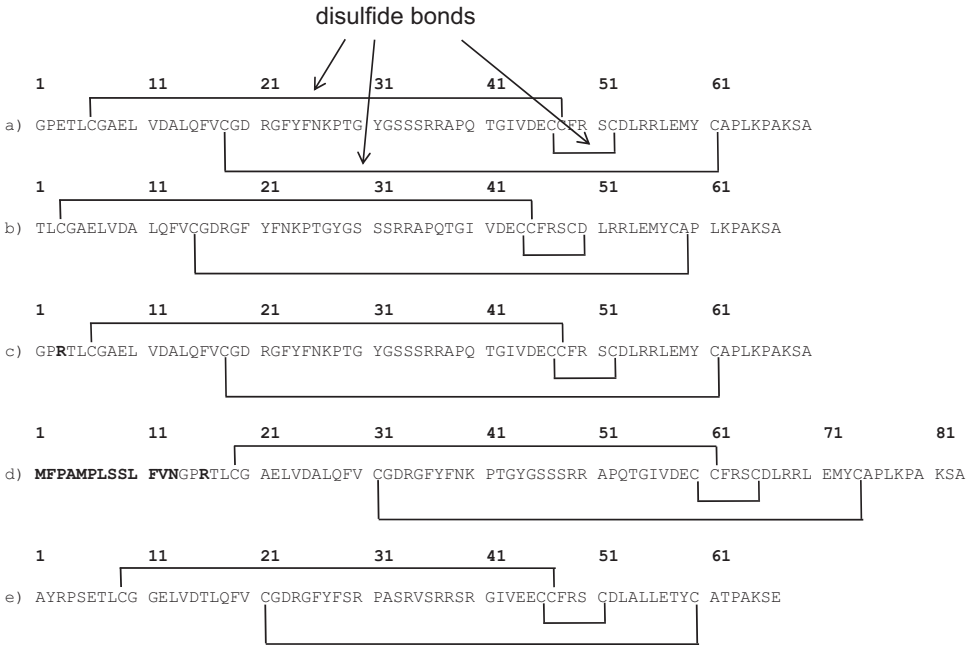

Fig. 5. Primary structures of IGF-1 (a), Des1-3-IGF-1 (b), R^3-IGF-1 (c), long-R^3-IGF-1 (d), and IGF-2 (e).

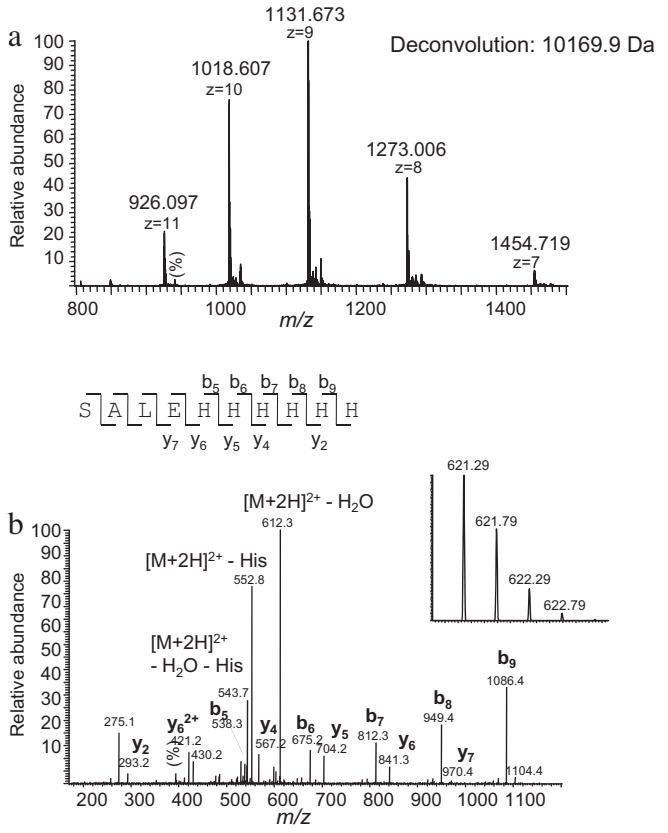

Fig. 6. Characterization of the C-terminally modified long-R^3-IGF-1 (additional amino acid residues written in bold): (a) full scan MS with deconvolution yielding the intact mass of 10169.9; (b) product ion mass spectrum of the tryptic peptide SALEHHHHHH cleaved from the C-terminus of the protein.

more appropriate analyte for doping control purposes. The simultaneous determination of urinary IGF-1 and IGF-2 as presented in this assay might provide supporting evidence in case of suspicion concerning a hGH misuse. As reported more than 2 decades ago [39], the (plasma) IGF-2/IGF-1 ratio might be significantly affected by a hGH application due to the differentially controlled releasing processes of both (IGF-2 and IGF-1) peptide hormones.

The necessity to comprehensively account for this class of substances has eventually been substantiated by frequent seizures of IGF-1 and related substances by customs and observations/intelligence concerning their misuse. An example of intended or accidental modification of long-R^3-IGF-1 was recently uncovered with the identification of a black-market compound comprising a C-terminal His_6-tag [40]. Found in the course of an anti-doping measure, an unlabeled vial was confiscated and its content studied by 1D/2D gel electrophoresis, top-down and bottom-up sequencing, and revealed the presence of long-R^3-IGF-1 bearing two linker amino acids and six histidine residues at the C-terminus (Fig. 6). The intact mass was determined with 10169.9 Da, and the characteristic peptide representing the C-terminus of the altered protein was sequenced by enzymatic hydrolysis and MS/MS experiments. In this context it is noteworthy that recombinant proteins are commonly furnished with His-tags during the expression process to facilitate subsequent purification; the tag is, however, usually attached to the N-terminus to enable a fast and simple removal by specific enzymes (e.g., for therapeutics). The use of C-terminally located His-tags is used predominantly in cases of biochemical studies requiring an intact/unmodified N-terminus; moreover, no C-terminally His-tagged peptide hormone has ever been approved for human use.

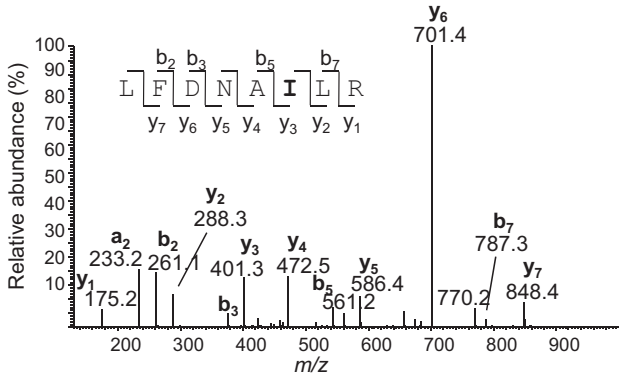

Fig. 7. Product ion mass spectrum of the doubly charged molecule $[M+2H]^{2+}$ of T2 at m/z 481.1 of the falsely translated recombinant hGH.

5. Human growth hormone (hGH)

Human growth hormone has long been suspected to be misused in elite sport, and recent adverse analytical findings as well as athletes' confessions corroborated this assumption [41,42]. In contrast to the above mentioned peptide hormones, the analysis of intact hGH from doping control samples by mass spectrometric approaches has not yet been accomplished; neither was the discrimination of natural from recombinantly produced hGH achieved due to the identity of the primary structures. Current tests utilize the different isoforms of hGH that are produced and secreted from the pituitary, which are not present in common pharmaceutical preparations that usually consist of the 22 kDa growth hormone variant only [43,44]. Consequently, in the so-called hGH isoform differential immunoassay, the 22 kDa isoform and non-22 kDa isoforms are quantitatively assayed by two separate analyses, and the ratio between the determined amounts is indicative for either a normal isoform distribution or an altered composition due to the administration of the recombinant formulation comprising the 22 kDa variant only. Recent studies however demonstrated the presence of up to 2% amino acid modification in recombinant GH preparations including deamidations (asparagines 149 and 152) and exchanges of methionines (positions 14, 125 and 170) by isoleucines [45]. The latter phenomenon is attributed to the false translation of the rare codon AGG in *Escherichia coli*, which might provide a viable target for sensitive doping control detection methods focusing on a specific target peptide. The product ion mass spectrum of the modified T2 obtained from Genotropin is shown in Fig. 7; the major issue of short half-life and low plasma as well as urine concentrations of hGH will however, remain.

6. Conclusions

Liquid chromatography–(tandem) mass spectrometry has become a central and invaluable tool in modern sports drug testing laboratories. Having been an established instrumentation for various low molecular mass compounds for more than a decade, its unique ability to enable the sensitive and specific detection and also quantitation of peptide hormones has not yet been fully adopted. Major reasons might be the efforts and costs for peptide hormone analysis by LC–MS(/MS), which are commonly higher than for low molecular mass analytes, and instruments are usually dedicated to peptide hormone analysis and rarely in combined use for small and large molecule measurements. However, first procedures targeting selected classes of peptide hormones such as insulins, GHRPs, IGFs, etc. have successfully been implemented and are available for routine doping controls.

Moreover, first comprehensive screening methods were developed that will become an integral part of future applications, which are seconded by dedicated and specifically sensitive methods targeting those minor differences between natural and recombinant peptidic drugs that allow the revelation of drug abuse.

Acknowledgments

The presented work was supported by the Federal Ministry of the Interior of the Federal Republic of Germany and the Manfred-Donike-Institute for Doping Analysis, Cologne. The authors wish to thank Dr. Laetitia Cravello and Dr. John Rontree (Waters, Manchester, UK) for enabling the ion mobility–MS(/MS) experiments.

References

[1] M. Thevis, Mass Spectrometry in Sports Drug Testing – Characterization of Prohibited Substances and Doping Control Analytical Assays, Wiley, Hoboken, 2010.
[2] M. Thevis, A. Thomas, W. Schänzer, Current role of LC–MS(/MS) in doping control, Anal. Bioanal. Chem., in press.
[3] M. Thevis, W. Schänzer, Identification and characterization of peptides and proteins in doping control analysis, Curr. Proteomics 2 (2005) 191–208.
[4] M. Thevis, W. Schänzer, Mass spectrometric identification of peptide hormones in doping control analysis, Analyst 132 (2007) 287–291.
[5] M. Thevis, A. Thomas, W. Schänzer, Insulin, Handb. Exp. Pharmacol. 195 (2010) 209–226.
[6] R.P. Green, A.S. Hollander, M. Thevis, A. Thomas, D.J. Dietzen, Detection of surreptitious administration of analog insulin to an 8-week-old infant, Pediatrics 125 (2010) e1236–e1240.
[7] A. Thomas, M. Kohler, W. Schänzer, P. Delahaut, M. Thevis, Determination of IGF-1 and IGF-2, their degradation products and synthetic analogues in urine by LC–MS/MS, Analyst 136 (2011) 1003–1012.
[8] M. Thevis, W. Schänzer, Current role of LC–MS(/MS) in doping control, Anal. Bioanal. Chem. 388 (2007) 1351–1358.
[9] R.I. Holt, P.H. Sonksen, Growth hormone, IGF-I and insulin and their abuse in sport, Br. J. Pharmacol. 154 (2008) 542–556.
[10] M. Thevis, A. Thomas, P. Delahaut, A. Bosseloir, W. Schanzer, Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography–tandem mass spectrometry, Anal. Chem. 78 (2006) 1897–1903.
[11] M. Thevis, A. Thomas, P. Delahaut, A. Bosseloir, W. Schänzer, Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity purification and liquid chromatography–tandem mass spectrometry for doping control purposes, Anal. Chem. 77 (2005) 3579–3585.
[12] A. Thomas, M. Thevis, P. Delahaut, A. Bosseloir, W. Schänzer, Mass spectrometric identification of degradation products of insulin and its long-acting analogues in human urine for doping control purposes, Anal. Chem. 79 (2007) 2518–2524.
[13] M. Thevis, A. Thomas, W. Schänzer, Mass spectrometric determination of insulins and their degradation products in sports drug testing, Mass Spectrom. Rev. 27 (2008) 35–50.
[14] A. Thomas, W. Schänzer, P. Delahaut, M. Thevis, Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled with high resolution/high accuracy mass spectrometry, Drug Test. Anal. 1 (2009) 219–227.
[15] P.H. Sonksen, Hormones and sport (insulin, growth hormone and sport), J. Endocrinol. 170 (2001) 13–25.
[16] S.M. Darby, M.L. Miller, R.O. Allen, M. LeBeau, A mass spectrometric method for quantitation of intact insulin in blood samples, J. Anal. Toxicol. 25 (2001) 8–14.
[17] A.D. Kippen, F. Cerini, L. Vadas, R. Stöcklin, L. Vu, R.E. Offord, K. Rose, Development of an isotope dilution assay for precise determination of insulin, C-peptide, and proinsulin levels in non-diabetic and Type II diabetic individuals with comparison to immunoassays, J. Biol. Chem. 272 (1997) 12513–12522.
[18] M.J. Chamberlain, L. Stimmler, The renal handling of insulin, J. Clin. Invest. 46 (1967) 911–919.
[19] B.H. Du, J. Eng, R.S. Yalow, Methods for concentration of urinary immunoreactive insulin, J. Korean Med. Sci. 1 (1986) 1–4.
[20] A.B. Kanu, P. Dwivedi, M. Tam, L. Matz, H.H. Hill Jr., Ion mobility-mass spectrometry, J. Mass Spectrom. 43 (2008) 1–22.
[21] E. Ghigo, E. Arvat, G. Muccioli, F. Camanni, Growth hormone-releasing peptides, Eur. J. Endocrinol. 136 (1997) 445–460.
[22] World Anti-Doping Agency, The 2011 Prohibited List, http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/To_be_effective/WADA_Prohibited_List_2011_EN.pdf (27-10-10).
[23] M. Okano, Y. Nishitani, M. Sato, A. Ikekita, S. Kageyama, Influence of intravenous administration of growth hormone releasing peptide-2 (GHRP-2) on detection of growth hormone doping: growth hormone isoform profiles in Japanese male subjects, Drug Test. Anal. 2 (2010) 548–556.
[24] S. Matschiner, Grenzwertig, RIVA Sportverlag, Wien, 2011.
[25] A. Thomas, S. Guddat, M. Kohler, O. Krug, W. Schänzer, M. Petrou, M. Thevis, Comprehensive plasma-screening for known and unknown substances in doping controls, Rapid Commun. Mass Spectrom. 24 (2010) 1124–1132.
[26] A. Thomas, S. Hoppner, H. Geyer, W. Schanzer, M. Petrou, D. Kwiatkowska, A. Pokrywka, M. Thevis, Determination of growth hormone releasing peptides (GHRP) and their major metabolites in human urine for doping controls by means of liquid chromatography mass spectrometry, Anal. Bioanal. Chem., in press.
[27] M. Okano, M. Sato, A. Ikekita, S. Kageyama, Determination of growth hormone secretagogue pralmorelin (GHRP-2) and its metabolite in human urine by liquid chromatography/electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 24 (2010) 2046–2056.
[28] M. Kohler, A. Thomas, H. Geyer, M. Petrou, W. Schanzer, M. Thevis, Confiscated black market products and nutritional supplements with non-approved ingredients analyzed in the Cologne Doping Control Laboratory 2009, Drug Test. Anal. 2 (2010) 533–537.
[29] J. Henninge, M. Pepaj, I. Hullstein, P. Hemmersbach, Identification of CJC-195, a growth-hormone-releasing peptide, in an unknown pharmaceutical preparation, Drug Test. Anal. 2 (2010) 647–650.
[30] A. Thomas, M. Kohler, J. Mester, H. Geyer, W. Schänzer, M. Petrou, M. Thevis, Identification of the growth-hormone-releasing peptide-2 (GHRP-2) in a nutritional supplement, Drug Test. Anal. 2 (2010) 144–148.
[31] I. Erotokritou-Mulligan, R.I. Holt, Insulin-like growth factor I and insulin and their abuse in sport, Endocrinol. Metab. Clin. North Am. 39 (2010) 33–43, viii.
[32] R.I.G. Holt, Detecting growth hormone abuse in athletes, Drug Test. Anal. 1 (2009) 426–433.
[33] D. Sinner, Anabole Steroide – Das Schwarze Buch, BMS Verlag, Gronau, 2007.
[34] M.A. Popot, A.R. Woolfitt, P. Garcia, J.C. Tabet, Determination of IGF-I in horse plasma by LC electrospray ionisation mass spectrometry, Anal. Bioanal. Chem. 390 (2008) 1843–1852.
[35] M. Bredehöft, W. Schänzer, M. Thevis, Quantification of human insulin-like growth factor-1 and qualitative detection of its analogues in plasma using liquid chromatography/electrospray ionisation tandem mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 477–485.
[36] S.S. de Kock, R.P. Rodgers, B.C. Swanepoel, Growth hormone abuse in the horse: preliminary assessment of a mass spectrometry procedure for IGF-1 identification and quantitation, Rapid Commun. Mass Spectrom. 15 (2001) 1191–1197.
[37] R.G. Kay, C. Barton, C.P. Velloso, P.R. Brown, C. Bartlett, A.J. Blazevich, R.J. Godfrey, G. Goldspink, R. Rees, G.R. Ball, D.A. Cowan, S.D. Harridge, J. Roberts, P. Teale, C.S. Creaser, High-throughput ultra-high-performance liquid chromatography/tandem mass spectrometry quantitation of insulin-like growth factor-I and leucine-rich alpha-2-glycoprotein in serum as biomarkers of recombinant human growth hormone administration, Rapid Commun. Mass Spectrom. 23 (2009) 3173–3182.
[38] S. Pan, R. Aebersold, R. Chen, J. Rush, D.R. Goodlett, M.W. McIntosh, J. Zhang, T.A. Brentnall, Mass spectrometry based targeted protein quantification: methods and applications, J. Proteome Res. 8 (2009) 787–797.
[39] A.T. Kicman, D.A. Cowan, Peptide hormones and sport: misuse and detection, Br. Med. Bull. 48 (1992) 496–517.
[40] M. Kohler, A. Thomas, K. Walpurgis, K. Terlouw, W. Schänzer, M. Thevis, Detection of His-tagged Long-R(3)-IGF-I in a black market product, Growth Horm. IGF Res. 20 (2010) 386–390.
[41] Associated-Press, in Associated Press, Tallin, 2011.
[42] Associated-Press, in Sports Illustrated, 2011.
[43] M. Bidlingmaier, J. Manolopoulou, Detecting growth hormone abuse in athletes, Endocrinol. Metab. Clin. North Am. 39 (2010) 25–32, vii.
[44] M. Bidlingmaier, C.J. Strasburger, Growth hormone, Handb. Exp. Pharmacol. 195 (2010) 187–200.
[45] F. Hepner, E. Cszasar, E. Roitinger, G. Lubec, Mass spectrometrical analysis of recombinant human growth hormone (Genotropin(R)) reveals amino acid substitutions in 2% of the expressed protein, Proteome Sci. 3 (2005) 1.