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Preliminary results for the development of a peptide screening method by means of LC-MS/MS

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Abstract

Bioactive peptides such as insulins, synthetic adrenocorticotrophic hormone (ACTH) analogue Synacthen, Gonadorelin (LHRH) and insulin-like growth factors (IGF) provide a reasonable potential for the misuse as performance enhancing agents and are prohibited in elite sports according to the list of banned substances established by the WADA. Currently, determination of these target analytes is possible by single assays only.

The present method provides results for a preliminary approach to determine various prohibited peptides occurring in urine (e.g. Gonadorelin, Humalog (Insulin Lispro), Apidra (Insulin Glulisine), Novolog (Insulin Aspart), Lantus (Insulin Glargine), Porcine Insulin, Bovine Insulin, IGF-1 etc.) in one screening procedure. The method enables the effective, highly sensitive and specific screening for several different target analytes that are simultaneously purified and analysed by means of immunoaffinity purification, subsequent liquid-chromatographic separation and high resolution / high accuracy mass spectrometric determination. Principally, the approach is extendable to any banned peptide, if adequate antibodies are available. At the present status of the project only a limited number of analytes were implemented in the method.

Introduction

The analysis of performance enhancing peptides or small proteins has reached an established status in the sports drug testing program of many doping control laboratories. Recently, a considerable number of methods to uncover the frequently reported misuse of peptides were published, and single assays were implemented into routine doping controls (1-18). Unfortunately, each class of these bioactive compounds (e.g. synthetic insulins, gonadorelin, Synacthen, IGF-1 etc.) requires a dedicated sample preparation procedure and, thus, the

workflow is, in comparison to commonly used screening methods for small molecules (e.g. stimulants, anabolic agents etc.) and less effective.

A simple combination of the different assays was hindered due to the heterogeneous character of the target analytes on the one hand and the low concentrations (in low fmol/mL) in urine on the other hand. Usually these challenges were handled by a highly specific and effective purification step using immunoaffinity approaches and additionally an enhanced chromatographic and mass spectrometric detection system composed of nanoscale UPLC coupled nano-electrospray ionisation and tandem mass spectrometry (6, 8, 9, 19).

The present study provides preliminary results for a combination of different assays resulting in a screening method for various different peptides without losing the necessary sensitivity or specificity. The approach is based on the simultaneous usage of different primary antibodies for the immunoaffinity step and combined purification with secondary antibody coated magnetic beads. Generally, the method is not limited to the presented analytes and expandable to further peptides or proteins if appropriate antibodies (AB) are available.

Chemicals and Reagents

Water, acetonitrile, trifluoroacetic acid and formic acid (all ultrapure for nano-liquid chromatography) were purchased from Biosolve (Valkenswaard, The Netherlands). Acetic acid (glacial), acetonitrile (analytical grade), sodium dihydrogenphosphate dihydrate (p.a.), disodium hydrogenphosphate dodecahydrate (p.a.), and sodium chloride (p.a.) were purchased from Merck (Darmstadt, Germany). Tris(carboxyethyl)phosphine hydrochloride (TCEP-HCl) was from Sigma (Deisendorf, Germany) and coated Dynal beads (anti-rabbit IgG, anti-mouse IgG) were obtained from Invitrogen (Karlsruhe, Germany). Polyclonal anti-ACTH antibodies (serum, anti-rabbit) and Anti-LHRH AB (serum, anti-rabbit) were purchased from Acris antibodies (Herford, Germany) and monoclonal Anti-Insulin AB (ascide fluid, anti mouse) and Anti-IGF-1 AB (polyclonal, Host: rabbit) were obtained from CER-groupe (Marloie, Belgium). Insulin analogues Humalog, Novolog, Apidra and Lantus were supplied by Eli Lilly (Indianapolis, IN), Novo Nordisk (Princeton, NJ), and Aventis (Kansas City, MO). Porcine insulin and bovine insulin were from Sigma (Deisendorf, Germany). LHRH reference substance was supplied as pharmaceutical formulation Kryptocur® by Sanofi-Aventis (Frankfurt, Germany). Synacthen Depot 1 mg was from Novartis Pharma (Bern, Switzerland). N-Acetyl-ACTH Fragment 1-17 used as internal standard (IS) was from Bachem (Bubendorf, Switzerland). Solid phase extraction cartridges OASIS HLB (60 mg, 3 mL) were bought from Waters (Eschborn, Germany). IGF-1, longR^3-IGF-1, R^3-IGF-1 and Des1-3-IGF-1 were

obtained from IBT-Biosystems (Reutlingen, Germany). All aqueous buffers and solutions were prepared in MilliQ water.

Sample preparation

The sample preparation procedure is schematically described in Fig. 1 and was described in detail earlier (19). Due to the fact that the presented data is preliminary and a final characterization and optimization of critical parameters are not finished yet, the detailed description will follow, but the main steps of the method will consist of solid phase extraction of 5 mL of urine, magnetic bead based immunoaffinity purification and subsequent LC-MS/MS detection. Details were also described earlier. (8, 9, 19)

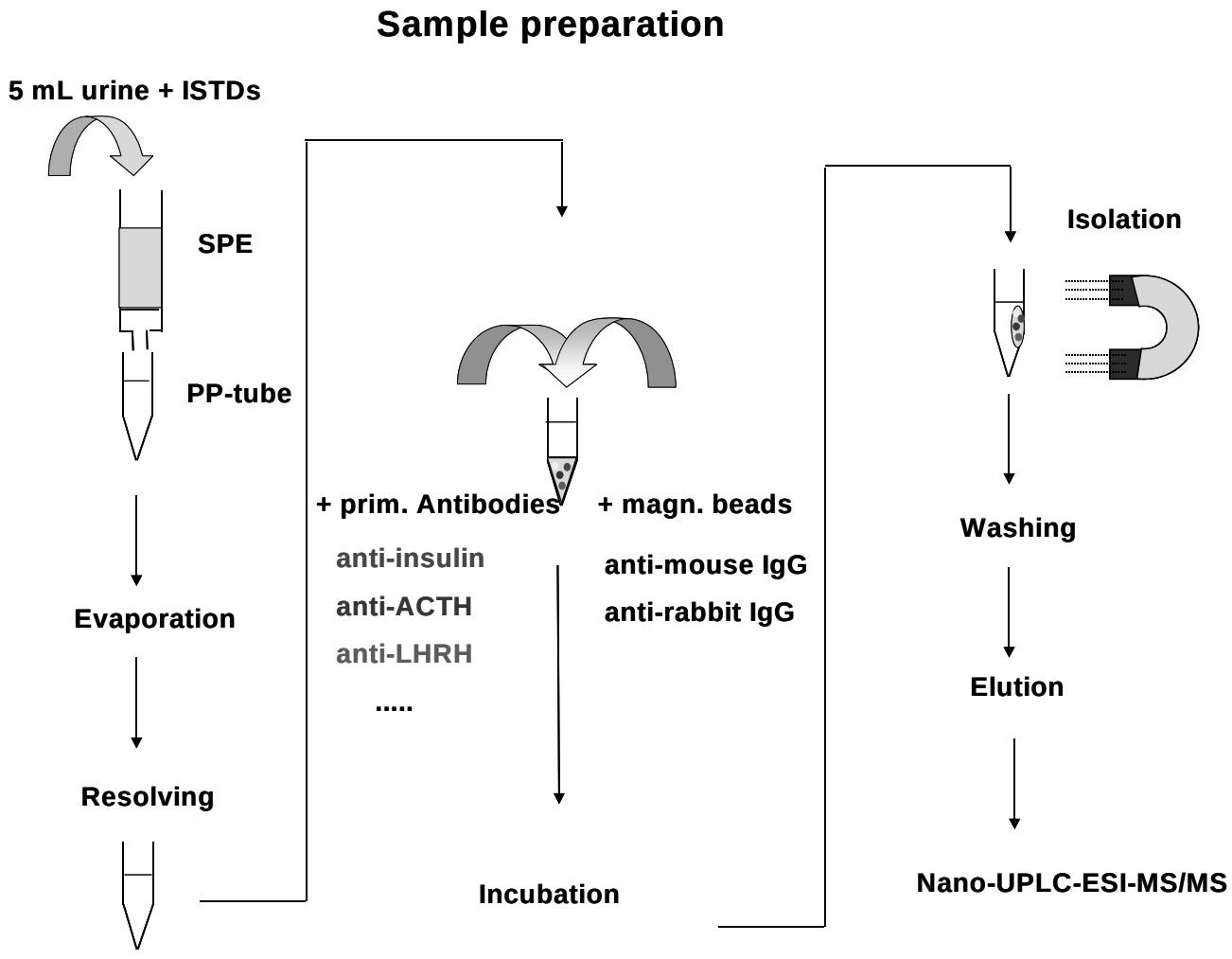

Figure 1: Scheme of the sample preparation procedure

Liquid chromatography / Mass spectrometry

Liquid chromatography was performed by means of a nanoUPLC (WATERS Acquity, Milford, USA) equipped with a WATERS BEH-130C_{18} (75 µm x 100 mm, 1.7 µm particle size) analytical column and a WATERS Symmetry C_{18} (180 µm x 20 mm, 5 µm particle size) trapping column. Solvent A consisted of ultrapure water acidified with 0.1 % of formic acid and solvent B was acetonitrile also containing 0.1 % of formic acid.

Pre-concentration of 1-2 µL (injection volume) of the prepared sample solution was achieved on the trapping column with 97 % of solvent A and a flow rate of 5 µL/min. After 3 min, the flow was diverted to the analytical column at 750 nL/min, and the gradient started with an isocratic step for 1 min with 97 % of solvent A. The percentage of organic solvent B increased to 100 % in 20 min, followed by a re-equilibration phase at starting conditions for 14 min. The overall runtime was 35 min. Mass spectrometry was performed on a Thermo LTQ-Orbitrap (Bremen, Germany) interfaced to the LC with a nanospray source (Thermo) equipped with a coated fused-silica emitter (New Objective, Ringoes, USA) using positive ionisation. Accurate mass measurement was ensured through calibration with the manufacturer's calibration mixture (consisting of caffeine, the tetrapeptide MetArgPheAla, and Ultramark 1621 mass spec standard) and the gas supply consisted of nitrogen (N_2-generator, CMC, Eschborn, Germany) and helium (purity 5.0). The ionisation voltage was set to 1.5 kV and the temperature of the transfer capillary was adjusted to 150 °C. Full scan spectra were recorded at a resolving power of 30,000 (full width half maximum, FWHM) in the analyser. Additionally, product ions of the most abundant precursors for Synacthen, LHRH and the different insulins were measured in the linear ion trap.

Method

The present study provides an analytical approach to isolate various target peptides from urinary specimens by solid phase extraction, followed by immunoaffinity purification with secondary antibody-coated magnetic beads and appropriate primary antibodies simultaneously from common doping control samples (Fig. 2).

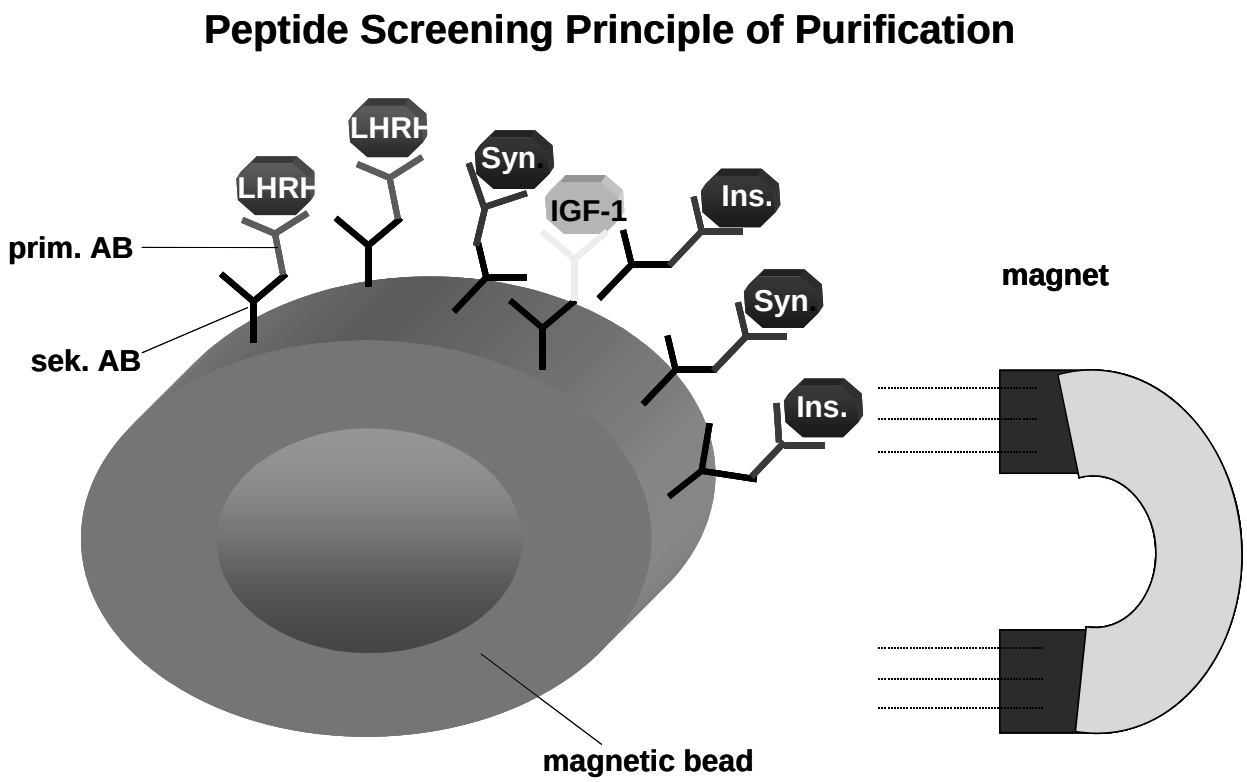

Figure 2: Scheme of purification procedure

Subsequently, unambiguous determination and identification of the purified peptides was performed after liquid chromatographic separation with tandem-mass spectrometry.

Results and Discussion

Concentrations for bioactive peptides in urine are rarely reported and mainly range in the low fmol/mL region due to efficient metabolic degradation and impaired renal clearance. (6, 8, 9) Nevertheless, the mass spectrometric determination of some performance enhancing peptides from urinary specimens after application is possible and was recently published (6, 8, 9, 19). Unfortunately, these methods were developed for single peptides or at least one class of peptides only and combination is hindered due to highly specific sample preparation procedures and heterogeneity of the analytes. In the present study different synthetic, animal and human insulins, Synacthen, IGF-1, longR^3-IGF-1 (and its degradation product Des1-10-longR^3-IGF-1) and LH-RH were sufficiently purified and determined in physiological relevant concentration levels (Fig. 3). Mass spectrometry was performed using a high resolution / high accuracy hybrid mass spectrometer after nano-UPLC separation and positive

nano-electrospray ionisation. Identification after collision-induced dissociation was enabled with diagnostic fragment ions from a top down approach fulfilling recommended criteria for identification of peptides (20).

Method validation for qualitative purposes was performed for each target analyte and LOD`s of 0.5 to 5 fmol/mL were achieved for endogenous, animal (porcine, bovine) and synthetic insulins (Humalog, Novolog, Apidra, Lantus), the synthetic ACTH-analogue Synacthen and the releasing hormone Gonadorelin.

Methods characteristics

Main performance characteristics of the method are summarized in Table 1.

	LOD, S/N>3		Precision at LOD	Recovery	Linearity			Precision	Specificity
	[fmol/mL]	[pg/mL]	[%]	[%]	slope	intercept	corr	[%]	
Synacthen	1	3	22	26	0.005	0.036	0.997	16	n. i. s.
Istd Ac-ACTH 1-17	-	-	-	-	-	-	-	-	-
Humalog	0.5	3	23	31	755.2	1.53	0.989	19	n. i. s.
Novolog	0.5	3	24	33	140.3	1.17	0.995	17	n. i. s.
Apidra	0.5	3	24	33	140.3	1.17	0.995	17	n. i. s.
Porcine Insulin	0.5	3	19	33	534.3	-0.83	0.998	11	n. i. s.
Lantus Metabolite	0.5	3	23	36	165.1	2.76	0.985	8	n. i. s.
Istd Bovine Insulin	0.5	3	-	-	-	-	-	-	-
LH-RH	5	5	-	105*	14.2	-17.35	0.998	15	n. i. s.
Istd DesPyr-LH-RH	-	-	-	-	-	-	-	-	-

* accuracy n. i. s.= no interfering signals

Table 1: Validation results

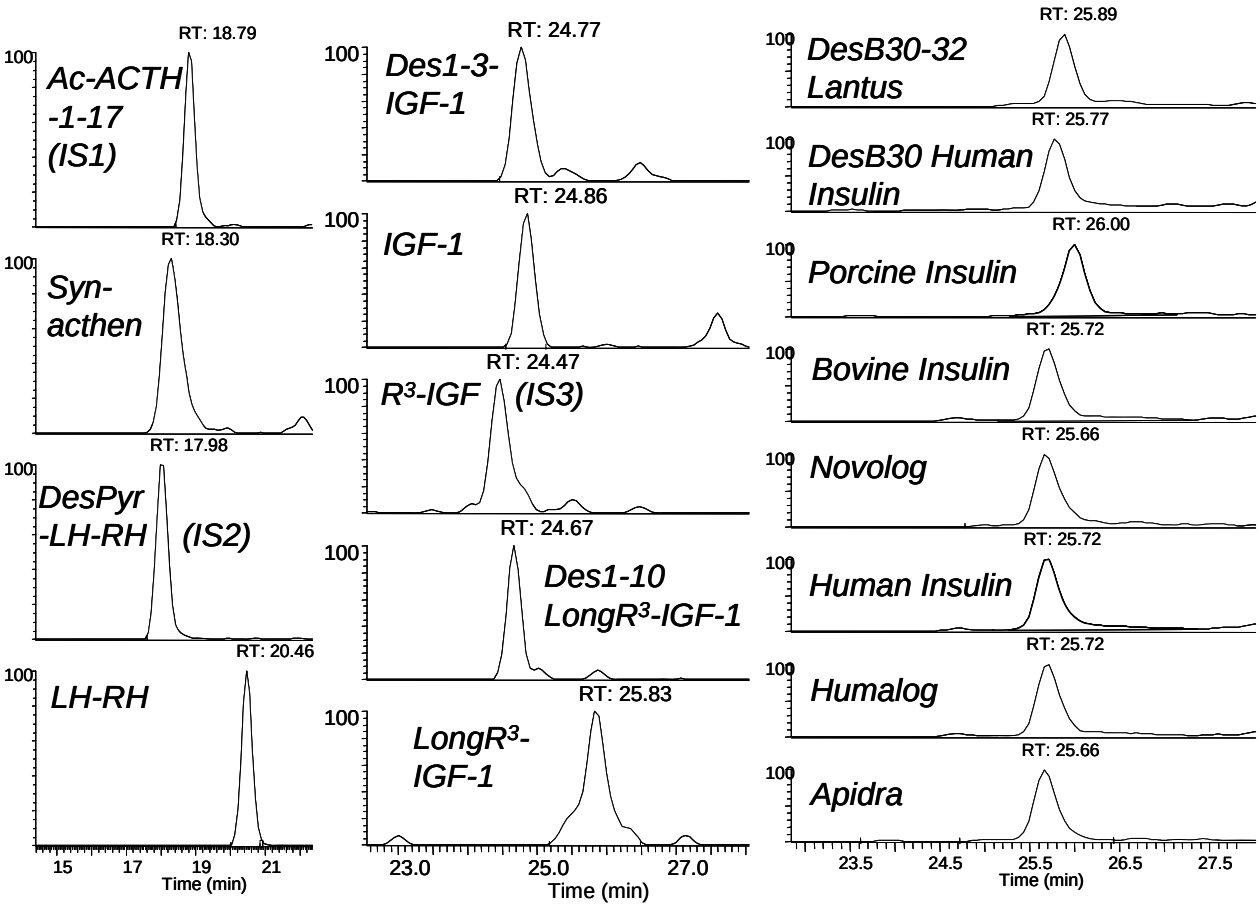

Figure 3: Extracted product ion chromatograms (LTQ) of a urine sample fortified with LH-RH, Synacthen, longR^3-IGF-1, R^3-IGF-1, Bovine insulin, Porcine insulin, Novolog, Apidra, Lantus Metabolite and Humalog in a range of 10 to 80 fmol/mL. IGF-1 and human insulin are of endogenous origin and indicate the required sensitivity of the assay.

Summary

The developed method enables the determination of different peptides in a fast (<12h), effective (>25 samples/day), sensitive (low fmol/mL) and highly specific manner. In addition to that, there are options for implementation of further peptides, if adequate antibodies are available and automation of each preparation step possible. All antibodies, chemicals and equipment are commercially available and the method will be expanded to more prohibited peptides or small proteins.

Future aspects

Screening procedures always represent the best compromise for all target analytes, but the optimal conditions for sample preparation and detection in this particular method were not finally evaluated to date. Thus, some critical preparation steps will be optimized.

Additionally, the method will be expanded to further peptides or small proteins with reasonable relevancy in doping control (2, 21). Finally, qualitative validation of all included targets will follow. Thus, a comprehensive screening procedure will be established and ideally close another gap in the list of prohibited substances of the WADA. In addition to the direct identification of banned compounds in urine, the absence of endogenous insulin or IGF-1 can also provide information regarding a possible manipulation (e.g. with proteases) of the specimen and induces further investigations of the urinary proteome (22).

References

1. Baume N, Steel G, Edwards T, Thorstensen E, Miller BF. (2008) No variation of physical performance and perceived exertion after adrenal gland stimulation by synthetic ACTH (Synacthen) in cyclists. *Eur J Appl Physiol*; **104**, 589-600.
2. Bredehöft M, Schänzer W, Thevis M. (2008) 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**, 477-485.
3. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W. (2005) 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**, 3579-3585.
4. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W. (2006) Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. *Anal Chem*; **78**, 1897-1903.
5. Thevis M, Bredehöft M, Geyer H, Kamber M, Delahaut P, Schänzer W. (2006) Determination of Synacthen in human plasma using immunoaffinity purification and liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*; **20**, 3551-3556.
6. Thevis M, Thomas A, Schänzer W. (2008) Mass spectrometric determination of insulins and their degradation products in sports drug testing. *Mass Spectrom Rev*; **27**, 35-50.
7. Thomas A, Thevis M, Delahaut P, Bosseloir A, Schänzer W. (2007) Mass spectrometric identification of degradation products of insulin and its long-acting analogues in human urine for doping control purposes. *Anal Chem*; **79**, 2518-2524.
8. Thomas A, Geyer H, Kamber M, Schänzer W, Thevis M. (2008) Mass spectrometric determination of gonadotrophin-releasing hormone (GnRH) in human urine for doping control purposes by means of LC-ESI-MS/MS. *J Mass Spectrom*; **43**, 908-915.

9. Thomas A, Schänzer W, Delahaut P, Thevis M. (2009) Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled to high resolution high accuracy mass spectrometry. *Drug Test. Analysis*; **1**, 219-227.
10. Abellan R, Ventura R, Palmi I, di Carlo S, Bacosi A, Bellver M, Olive R, Pascual JA, Pacifici R, Segura J, Zuccaro P, Pichini S. (2008) Immunoassays for the measurement of IGF-II, IGFBP-2 and -3, and ICTP as indirect biomarkers of recombinant human growth hormone misuse in sport. Values in selected population of athletes. *J Pharm Biomed Anal*; **48**, 844-852.
11. Cowan DA, Bartlett C. (2009) Laboratory issues in the implementation of the marker method. *Growth Horm IGF Res*; **19**, 357-360.
12. Holt RI, Bassett EE, Erotokritou-Mulligan I, McHugh C, Cowan D, Bartlett C, Sonksen PH. (2009) Moving one step closer to catching the GH cheats: The GH-2004 experience. *Growth Horm IGF Res*; **19**, 346-351.
13. Holt RI, Erotokritou-Mulligan I, Sonksen PH. (2009) The history of doping and growth hormone abuse in sport. *Growth Horm IGF Res*; **19**, 320-326.
14. Holt RI, Sonksen PH. (2008) Growth hormone, IGF-I and insulin and their abuse in sport. *Br J Pharmacol*; **154**, 542-556.
15. Kicman AT, Cowan DA. (1992) Peptide hormones and sport: misuse and detection. *Br Med Bull*; **48**, 496-517.
16. Sonksen PH, Holt RI. (2009) GH & IGF Research issue on doping with growth hormone. *Growth Horm IGF Res*; **19**, 283-284.
17. Kicman AT, Miell JP, Teale JD, Powrie J, Wood PJ, Laidler P, Milligan PJ, Cowan DA. (1997) Serum IGF-I and IGF binding proteins 2 and 3 as potential markers of doping with human GH. *Clin Endocrinol (Oxf)*; **47**, 43-50.
18. Sonksen PH. (2001) Insulin, growth hormone and sport. *J Endocrinol*; **170**, 13-25.
19. Thomas A, Kohler M, Schänzer W, Kamber M, Delahaut P, Thevis M. (2009) Determination of Synacthen in urine for sports drug testing by means of nano-ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*; **23**, 2669-2674.
20. Thevis M, Loo JA, Loo RR, Schänzer W. (2007) Recommended criteria for the mass spectrometric identification of target peptides and proteins (<8 kDa) in sports drug testing. *Rapid Commun Mass Spectrom*; **21**, 297-304.
21. Kohler M, Thomas A, Walpurgis K, Terlouw K, Schänzer W, Thevis M. (2010) Detection of His-tagged Long-R(3)-IGF-I in a black market product. *Growth Horm IGF Res*.
22. Thomas A, Kohler M, Walpurgis K, Schänzer W, Thevis M. (2009) Proteolysis and autolysis of proteases and the detection of degradation products in doping control. *Drug Test. Analysis*; **1**, 65-72.