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Detection of recombinant growth hormone in plasma by a 2D-PAGE method: an update

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Abstract

Human growth hormone (GH) is prohibited in sports by the World Anti-Doping Agency (WADA) and two different approaches for a doping control method are currently discussed. The Strasburger test is presently used as doping control method and the marker test measuring the indirect markers IGF-I and Procollagen III by Sönksen et al. is discussed as complementation. The assay that is presented here could be used as confirmation assay in combination with the Strasburger test.

The method based on 2D-PAGE was validated and a reference population was analyzed to establish a discrimination limit. As a proof of principle, patient samples were prepared and were clearly positive 2.5 h after s.c. injection. After 12-16 h, three out of the five samples still had values higher than the discrimination limit. Four variants are detected in endogenous samples (22 kDa GH, splice variant, phophorylated GH, and glycosylated GH) and the mass spectrometric identification of the glycosylated variant is presented here. For comparison and to test the combination of the Strasburger test and the 2D-PAGE method the patient samples were analyzed with both tests. The results showed that a combination of both tests can improve the overall performance of growth hormone analysis in doping control. Furthermore an antibody alternative was tested and the sensitivity of the 2D-PAGE method was shown to be improved by the use of a secondary AMDEX™ antibody.

Introduction

Human endogenous growth hormone (hGH) is one of the pituitary hormones and it is released from the pituitary in a pulsatile manner depending on several external or endogenous stimuli. While cadaver pituitary growth hormone was originally used to treat children with dwarfism, recombinant growth hormone is available for medicinal treatment since the late 1980th and replaced the pituitary protein for therapy. This has also increased the possibilities for misuse

of growth hormone because of a better availability but also allowed the development of doping control methods because recombinant and endogenous growth hormone are not completely identical. Beside dwarfism, growth hormone is also used to treat growth hormone deficiency of adults. The replacement in adults is necessary because growth hormone exerts several metabolic functions and plays an important role in fat and muscle metabolism which makes it especially interesting for athletes. While recombinant growth hormone consists of one variant only, namely the unmodified 22 kDa growth hormone, endogenous growth hormone is a combination of several different variants.[1, 2] Modifications that were already identified are e.g. phosphorylation[3], acylation, as well as proteolytic cleavage[4] and dimerization[5, 6]. Additionally, glycosylation[7] was suspected after enzymatic and chemical deglycosylation experiments. Although the unmodified 22 kDa growth hormone accounts for about 90% of total growth hormone in the body, the identification of modifications and variants may help to discover further functions of growth hormone or explain the metabolic effects. The currently used doping control method[8, 9] is also based on the measurement of different variants and the doping control confirmation method by 2D-PAGE detects discrete and identified growth hormone variants.[10]

The present study demonstrates the completion of the validation of the 2D-PAGE method and the establishment of a preliminary discrimination limit. Additionally, a glycosylated growth hormone variant is identified by mass spectrometry techniques and improvements of the doping control method concerning the antibody used and the sensitivity of the method are presented.

Materials and Methods

Materials: The recombinant growth hormone used was Genotropin™ from Pharmacia (Karlsruhe, Germany), and human placental lactogen (Swissprot ID: Q6PF11) used as internal standard was from Serotec (Düsseldorf, Germany). Secondary antibody-coupled magnetic beads (anti-rabbit IgG antibody produced in sheep) as well as 12% Bis-Tris SDS gels, MOPS (3-(N-morpholino)propanesulfonic acid) running buffer and lithium dodecyl sulphate (LDS) sample buffer were purchased from Invitrogen (Karlsruhe, Germany). The primary antibody (polyclonal, from rabbit) was purchased from Acris Antibodies (GTX72790, Hiddenhausen, Germany) and the alternative antibody from CER groupe (Centre d´Economie Rurale, Marloie, Belgium). IPG (immobilized pH gradient) strips (18 cm, pH 4.7-5.9, 24 cm pH 4.5-5.5, pH 4-7) were from Bio-Rad (Munich, Germany). Immobilon-P membranes (PVDF membranes) were bought from Millipore (Billerica, MA, USA) and the detection system

including the blocking reagent (ECL Advance) as well as the secondary AMDEX antibody was from GE Healthcare (Munich, Germany). The Coomassie Blue stain was purchased from Pierce (Rockford, IL, USA). The secondary anti-rabbit IgG antibody (produced in goat, A9169) as well as Dithiothreitol (DTT), acrylamide (both analytical grade), bovine serum albumin (BSA, 97 %) and all buffer ingredients (electrophoresis grade) were from Sigma (Deisendorf, Germany), and ethanol (96 %) was from VWR International (Darmstadt, Germany).

Blood samples: Blood samples were taken from healthy volunteers or patients that gave their written consent to participate in the study and ethical approval was obtained from the local ethical committee. Blood samples were collected into EDTA tubes and centrifuged for 10 min at 2700 *g*. Plasma (1 mL) was transferred to a 2 mL Eppendorf tube and samples were frozen at –20°C until analysis. To determine reference values for the detected isoforms of endogenous growth hormone, 33 samples from different volunteers were prepared and analyzed (age 14-34 years, 16 male, 17 female). Samples from patients that apply recombinant growth hormone (Genotropin™ from Pharmacia (Karlsruhe, Germany), Humatrope™ from Lilly (Bad Homburg, Germany) or Omnitrope™ from Sandoz (Ismaning, Germany)) were provided by the Endokrinologikum Hamburg, Germany. Samples of five patients (age 7-14 years, 3 male, 2 female) that suffer from either growth hormone deficiency or idiopathic short stature were analyzed. The daily injection amount was approximately 50 µg/kg body weight. Blood samples were taken prior to and approximately 2.5 hours after subcutaneous injection.

Pituitary samples: Pituitary glands were provided by the Institute of Legal Medicine, University Hospital Hamburg-Eppendorf, Germany. They were homogenized in 2 mL of multichaotropic sample solution (MCSS, 7.7 M urea, 2.2 M thiourea, 4.4 % CHAPS, 44 mM Tris) by manual treatment with a micromortar and were continuously sonicated for 30 min. After centrifugation (5 min, 9000 *g*), the supernatant was transferred to a new tube and frozen at -20 °C until analysis.

Methods:

OFFGEL isoelectric focussing and LC/MS for identification of the glycosylation

For OFFGEL fractionation of pituitary samples, 500 µL of pituitary extract were washed with water in a centrifugal filter (molecular weight cut-off 5 kDa) and diluted in OFFGEL stock solution as recommended by the supplier (Agilent Technologies, Waldbronn, Germany). For fractionation, 24 cm IPG strips with a pH gradient of 4.5-5.5 or 4-7 and the 24 well frame set

were used. Samples were run as recommended with a maximum current of 50 µA per strip to a total of at least 50,000 Vh, and 50-150 µL of the supernatant was recovered from each well. For visualization of the proteins in the fractions, SDS gels were prepared using 20 µL of each fraction, adding 7 µL of LDS sample buffer and 3 µL of DTT (1M) and heating 10 min at 70 °C. After the SDS-PAGE (125 V, 100 min), gels were stained with Coomassie Blue. Fractions of interest were concentrated in a centrifugal filter (cut-off 5 kDa) and washed with water prior to further separation by capillary LC and identification of intact modified growth hormone isoforms by high resolution/high accuracy mass spectrometry (*vide infra*).

The capillary LC was an Agilent 1100 instrument with a Zorbax 300 SB - C18 precolumn (3.5 µm, 50 x 0.3 mm) and a Zorbax 300 SB - C18 analytical column (5 µm, 5 x 0.3 mm). Solvents were 0.1 % acetic acid, 0.01 % TFA (solvent A) and 80 % acetonitrile, 0.1 % acetic acid, 0.01 % TFA (solvent B). The following gradient program was applied: 0-2 min 95 % A, 2- 25 min 60 % A, 25-34 min 3 % A, 34-44 min 3 % A, re-equilibration 16 min 95 % A. Ionization voltage was set to 3.5 kV in positive mode, the maximum fill time for the orbitrap was 5000 ms and the resolving power was 100,000 at *m/z* 400 (FWHM). Prior to mass spectrometry measurements, mass calibration of the instrument was performed with the manufacturer´s calibration mixture.

For the detection of typical carbohydrate fragments, MS/MS spectra of intact modified growth hormone were recorded on an Applied Biosystems Qtrap 4000 mass spectrometer (Foster City, CA, USA) after separation on an Agilent 1100 liquid chromatograph (Palo Alto, CA, USA). The LC was equipped with a Zorbax StableBond guard column (1 mm x 17 mm, 5 µm) and a Zorbax 300SB-C18 analytical column. Solvents used were 1 % acetic acid/0.1% TFA (A) and acetonitrile/solvent A (80:20) (B) with the following gradient: 95 % A, 0-25 min 15 % A, 10 min reeqilibration. The collision energy for MS/MS experiments was set to 40 eV.

<u>Doping control method:</u> The method for the analysis of growth hormone from plasma samples allows the preparation of six samples at once and takes two and a half days for isolation of growth hormone from plasma, 2D-PAGE and immunostaining.

<u>Isolation of growth hormone from plasma:</u> Growth hormone was isolated from 1 mL of plasma by magnetic bead separation. A volume of 75 µL of magnetic beads coupled to a secondary anti-rabbit IgG antibody and 2.5 µL of polyclonal anti-GH antibody (1 mg/mL) were added to 1 mL of plasma. Additionally, 0.2 µg of human placental lactogen were added as internal standard. Samples were incubated with rotating on an overhead shaker over night

at 4°C, washed two times with PBS (140 mM NaCl, 8 mM Na_2HPO_4, 1.7 mM NaH_2PO_4) and analytes were eluted from the beads with two times 130 µL of MCSS.

Separation by 2D-PAGE and visualization: Proteins were prepared for the isoelectric focusing by reducing disulfide bonds with 15 µmol of DTT (45 min room temperature (RT)) and derivatizing cysteine residues with acrylamide (45 µmol, 45 min RT). After reaction of the acrylamide excess with further 30 µmol of DTT (10 min at RT) and rehydration loading of the samples to the IPG strips, isoelectric focusing was performed over night in an Ettan IPGphor 3 (GE Healthcare) with the following voltage gradient program: 500 V, 1 h; 500-1000 V, 2 h; 1000 to 10000 V, 3 h; 10000 V for 4 h (total: 57000 Vh). The maximum current per strip was set to 75 µA and the temperature was 20°C. After additional 15 min at 10000 volts in the morning for refocusing, strips were equilibrated two times for 10 min in LDS sample buffer. Strips were cropped to a pH range of approximately 5.0-5.5 and applied to 8 cm, 12% Bis-Tris gels (XCell6 (Invitrogen), 125 V, 90 min).

After semidry blotting to a PVDF membrane (TE77PWR (GE Healthcare), 1 mA/cm^2, 45 min, blotting buffer: 39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% ethanol) the membrane was blocked in blocking solution for 1 h. Subsequently, the primary antibody was diluted 1:2000 in blocking solution (1 h incubation), and after washing three times for 10 min with $PBST_{ECL}$ (100 mM NaCl, 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 0.2% Tween 20) the membrane was incubated with secondary antibody for one hour (1:7500). Detection was performed using ECL Advance chemiluminescent reagent.

The alternative primary antibody from CER groupe was used in a 1:1000 dilution for the detection and 5 µl were used in the immunoaffinity purification.

The secondary AMDEX™ antibody was applied in a 1:10000 dilution and also incubated for one hour.

Data analysis: Pictures of plasma sample blots were recorded with a FluorChem CCD camera using Alpha Ease FC 3.0 software (both Biozym, Oldendorf, Germany) and analyzed with Image Master 2D Platinum 6.0 software (GE Healthcare), which enabled the calculation of spot volumes. Spots were encircled using the spot editing tool and spot volumes were calculated automatically. Circles were chosen as narrow around the spots as possible to avoid the necessity of a background subtraction. Volume values were normalized to the volume of the internal standard.

Results and Discussion

Figure 1 shows the principle of the 2D-PAGE doping control method. The membrane from the sample containing endogenous growth hormone shows 4 spots (22 kDa GH (Spot 1), 20 kDa Splice variant (Spot 2), phophorylated GH (Spot 3) and glycosylated GH (Spot 4)) while a membrane of a sample containing recombinant growth hormone only shows spot 1. The internal standard used is human placental lactogen.[11, 12]

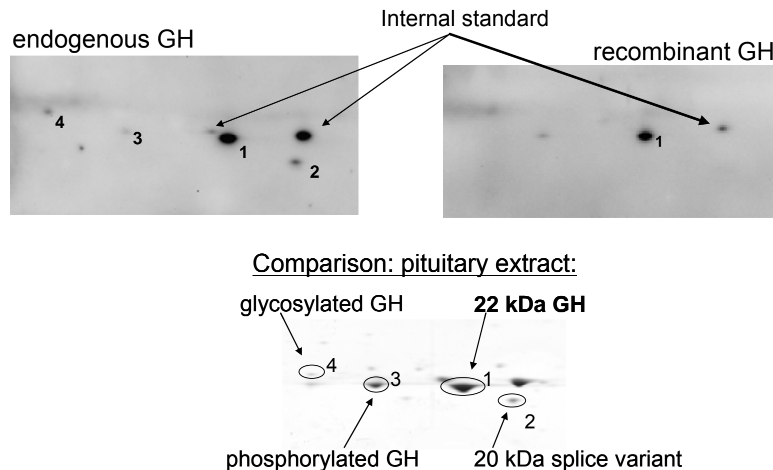

Figure 1: comparison of a membrane from plasma samples with endogenous and recombinant GH. In the lower part of the figure a Comassie Blue stained gel (pituitary extract) is shown with the variants identified by mass spectrometry.

<u>Identification of the glycosylated GH variant by mass spectrometry techniques</u>

While the identification of spots 1-3 was described in detail before [13], the glycosylation in spot 4 was identified in this study. The identification of the glycosylation was accomplished after OFFGEL-IEF fractionation and *Top-Down* mass spectrometric analysis (Figure 2). Deconvolution of the two species in the spectrum (Figure 2) yielded masses of the proteins of 23062.5 Da for the modified variant and 22115.1 Da for the unmodified GH. Subtraction then yielded a mass of 974.4 Da for the modification. MS/MS experiments on a API4000 QTrap yielded characteristic carbohydrate fragments of m/z 274, 292 and 366 being typical for sialic acid, sialic acid-water and a HexHexNac residue (data not shown).[11] Summation of the theoretical masses of the fragments and the unmodified growth hormone yielded exactly the mass of the modified variant as determined by deconvolution from Figure 2. The database search for prediction of possible O-glycosylation site yielded one result at Threonine 60.[14] This is consistend with Bottom-Up analysis with trpysin or Glu-C, where the peptide including threonine 60 was not present in the modified spot.

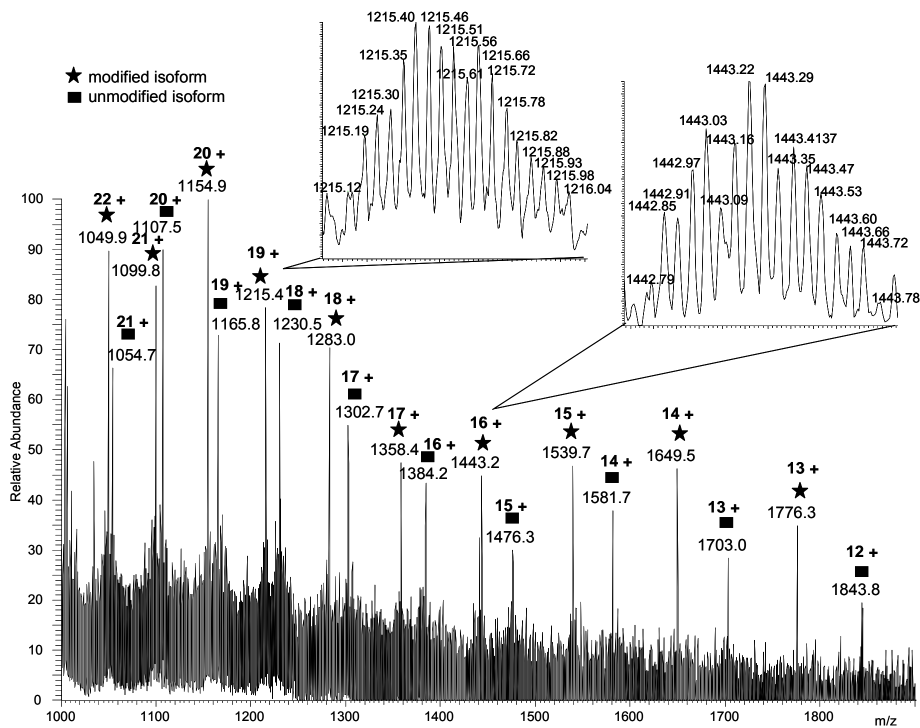

Figure 2: Mass spectrum of modified (glycosylated) and unmodified GH recorded on an LTQ Orbitrap

The identification of all variants (including the glycosylation) that are detected in the doping control method makes the assay especially powerful and unusual proportions of variants will easily be detected without the problem of generating false suspicious results because discrete variants can be detected and distinguished here.

Doping control method:

The development and validation of the doping control method was described earlier [10, 12]. Additionally, it was proven that the method works with plasma as well as serum samples, which is important if used as complementation of current tests that necessitate the use of serum. After successful validation of the method, a reference population was analyzed to establish a preliminary discrimination limit. For that purpose normalized spot volumes were calculated (Spot volume (GH spot)/ spot volume (internal standard spot)) and the the NSVs of the different variant are shown as bars in Figure 3. Samples were separated into groups for samples without GH spot, samples with 1 GH spot (22 kDa GH), samples with 2 GH spots and samples with 4 GH spots. Calculation confidence intervals (99,9%) lead to a preliminary discrimination limit for the NSV of the unmodified 22 kDa spot of 0.52.[12] If a sample has a higher spot volume than 0.52 and only shows 1 spot but no 20 kDa splice variant, it is suspicious to contain recomibinant growth hormone. The chosen discrimination limit was proven by analysis of patient samples (Figures 3 and 4) and 2.5 hours after injection all

samples were clearly positive. 12-16 hours after injection three out of the five samples still had NSVs > 0.52 and showed only that one spot.

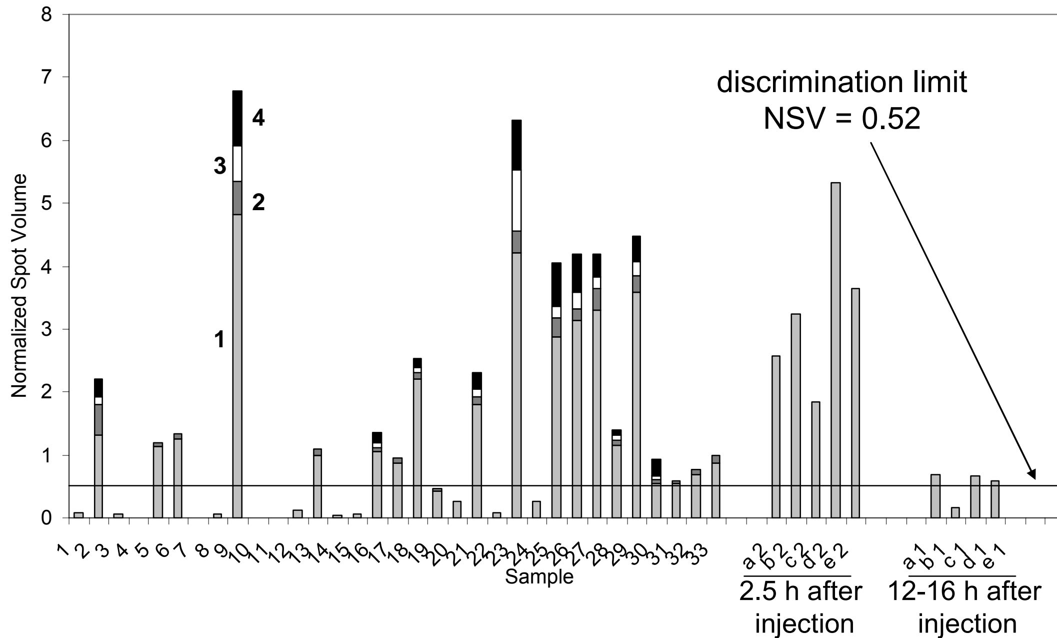

Figure 3: Normalized spot volumes of the different variants for a reference population (sample 1-33) and patient samples (samples a-e). Bars 1-4 represent the NSVs of spots 1-4 (Figure 1). The horizontal line indicates the discrimination limit for the NSV of the unmodified 22 kDa spot (spot 1 from Figure 1).

Comparison of the results of the patient samples with the currently used luminescence immunoassay (LIA, Strasburger test) showed that Kit 1 also recognized all samples 2.5 hours after injection to contain recombinant growth hormone but for Kit 2 only one sample was above the decision limit (see Figure 4). Interestingly, some concentrations were much higher in the LIA than with a regular enzyme-linked immunosorbent assay (ELISA) for GH quantification (e.g. sample d and e). The reasons for those differences are not clear but may indicate a cross reaction of the antibody. It has to be noted that the patients were 7-14 year old children and the luminescence assay is may not be tested for this population. Nevertheless, especially those uncertainties could be avoided if the 2D-PAGE method is used in combination with the immunoassay because it detects discrete variants and cross reactions would produce an additional spot and not enhance the signal of one of the spots as it is for the immunoassay.

		B-Kit				A-Kit					
		conc rec [ng/mL]	conc pit [ng/ml]	rec/ pit		conc rec [ng/mL]	conc pit [ng/mL]	rec/ pit		conc ELISA [ng/mL]	NSV 2D-PAGE
~2.5 h after injection											
a	w	8.51	3.83	2.22		8.67	4.99	1.74		9.3	2.57
b	m	11.23	5.1	2.20		10.33	6.17	1.67		9.7	3.24
c	m	5.93	2.3	2.58		5.35	3.04	1.76		7.2	1.83
d	m	26	13.46	1.93		23.92	17.69	1.35		11.3	5.32
e	m	28.54	20.74	1.38		8.75	8.34	1.05		11.3	3.64
12-16 h after injection											
a	w	1.5	0.75	2.00		1.24	0.88	1.41		1.6	0.69
b	m	0.91	1.72	0.53		0.77	1.46	0.53		0.92	0.16
c	m	1.4	0.55	2.55		1.53	0.84	1.82		1.85	0.66
d	m	0.77	0.88	0.88		0.69	0.85	0.81		0.97	0.59
e	m	0.04	0.09	0.44		n.a.	n.a.	n.a.		0.07	< LOD
decision limits:				w = 1.19 m = 1.64				w = 1.68 m = 2.17			0.52

Figure 4: Comparison of the results from the 2D-PAGE assay and the currently used luminescence immunoassay. Dark grey fields show positive results.

Due to the problem that polyclonal antibodies may run out of stock if bought from any commercial source, another antibody obtained from CER groupe was tested after assuring that this antibody will be available for an adequate time period or number of samples. The antibody was shown to detect the same variants as the "old" antibody with a comparable sensitivity and after an additional validation this antibody could be also used for the 2D-PAGE assay.

The main draw-back of the 2D-PAGE method in its original setup[12] may be the sensitivity (LOD 0,25 ng/ml, discrimination limit ~ 1 ng/ml). An increased sensitivity was achieved by using a different secondary antibody which enhances the signal because it is bound to a bigger number of horse radish peroxidase molecules (AMDEX) and more detailed studies will be done to implement this optimization of the procedure.

Concluding remarks

The study completes the method development of the confirmatory rGH 2D-PAGE assay. Additionally, sensitivity can be improved which will be systematically tested in future. In the case of antibody shortage from the commercial source, polyclonal antibodies from CER

groupe detect the same variants with a similar sensitivity which allows the exchange of “old” antibody after an additional validation which is planned for a future study.

The 2D-PAGE assay is a powerful complementation of the immunoassay currently used and should be taken into account as confirmation method as soon as the last optimizations on sensitivity and antibody are finished. It excludes problems concerning unknown cross reactions due to the measurement of discrete variants and the use of the internal standard allows the monitoring of the sample preparation.

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References

1. Baumann G. (1991) Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. *Endocr Rev.***12**, 424-449.
2. Thevis M, Schänzer W. (2005) Identification and Characterization of Peptides and Proteins in Doping Control Analysis. *Currents Proteomics.***2**, 191-208.
3. Zhan X, Giorgianni F, Desiderio DM. (2005) Proteomics analysis of growth hormone isoforms in the human pituitary. *Proteomics.***5**, 1228-1241.
4. Spolaore B, Polverino de Laureto P, Zambonin M, Fontana A. (2004) Limited proteolysis of human growth hormone at low pH: isolation, characterization, and complementation of the two biologically relevant fragments 1-44 and 45-191. *Biochemistry.***43**, 6576-6586.
5. Nagatomi Y, Ikeda M, Uchida H, Wada M, Kobayashi H, Hashimoto Y, Mabuchi K, Hayakawa M, Kusuhara N, Honjo M. (2000) Reversible dimerization of 20 kilodalton human growth hormone (hGH). *Growth Horm IGF Res.***10**, 207-214.
6. Lewis UJ, Peterson SM, Bonewald LF, Seavey BK, VanderLaan WP. (1977) An interchain disulfide dimer of human growth hormone. *J Biol Chem.***252**, 3697-3702.
7. Haro LS, Lewis UJ, Garcia M, Bustamante J, Martinez AO, Ling NC. (1996) Glycosylated human growth hormone (hGH): a novel 24 kDa hGH-N variant. *Biochem Biophys Res Commun.***228**, 549-556.
8. Bidlingmaier M, Wu Z, Strasburger CJ. (2000) Test method: GH. *Baillieres Best Pract Res Clin Endocrinol Metab.***14**, 99-109.
9. Bidlingmaier M, Suhr J, Ernst A, Wu Z, Keller A, Strasburger CJ, Bergmann A. (2009) High-Sensitivity Chemiluminescence Immunoassays for Detection of Growth Hormone Doping in Sports. *Clin Chem*.
10. Kohler M, Püschel K, Sakharov D, Tonevitskiy A, Schänzer W, Thevis M. (2008) Detection method for recombinant human growth hormone based on 2D-PAGE. In: Schänzer W, Geyer H, Gotzmann A, Mareck U, eds. *Recent advances in doping analysis*. Köln.pp.
11. Kohler M, Thomas A, Püschel K, Schänzer W, Thevis M. (2009) Identification of Human Pituitary Growth Hormone Variants by Mass Spectrometry. *J Proteome Res*.
12. Kohler M, Puschel K, Sakharov D, Tonevitskiy A, Schanzer W, Thevis M. (2008) Detection of recombinant growth hormone in human plasma by a 2-D PAGE method. *Electrophoresis.***29**, 4495-4502.
13. Kohler M, Püschel K, Schänzer W, Thevis M. (2007) Mass spectrometric identification of human pituitary growth hormone variants. In: Schänzer W, Geyer H, Gotzmann A, Mareck U, eds. *Proceedings of the 24th Cologne Workshop on Dope Analysis*. Cologne.pp. 21-30
14. Center for Biological Sequence Analysis DoSB, Technical University of Denmark. (access 02.10.08) http://www.cbs.dtu.dk/services/NetOGlyc/. *Lyngby, Denmark*.