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Detection method for recombinant human growth hormone based on 2D-PAGE

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Abstract

Human growth hormone (GH) has several central metabolic functions including bone growth in childhood and its anabolic and lipolytic effects in particular are assumed reasons for the abuse of GH by athletes. Human endogenous GH consists of a main 22 kDa variant and several isoforms. In contrast, recombinant GH consists of only one variant being identical to the main endogenous isoform. The method presented here separates different isoforms by 2D-PAGE after isolation of growth hormone from plasma using an immunoaffinity purification system. While samples containing endogenous GH yield up to four isoforms, samples with recombinant GH are supposed to contain the main 22 kDa spot only. External stimuli like exercise trigger the release of growth hormone from the pituitary. It was shown that the release is detected by the presented method as an increase in all isoforms. This approach detects discrete isoforms of GH from plasma and discriminates endogenous GH from its recombinant analog which makes it a useful approach for doping control purposes.

Introduction

Human endogenous growth hormone (hGH) is a protein composed by 191 amino acids with a molecular weight of 22 kDa and is produced in the pituitary gland. Alternative splicing results in a smaller isoform of 20 kDa missing the amino acids 32-46[1], and different posttranslational modifications such as phosphorylation [2], acylation, glycosylation [3] as well as proteolytic cleavage [4] and dimerization [5, 6] lead to a large heterogeneity [7, 8]. Due to a pulsatile release of growth hormone from the pituitary, circulating amounts vary widely within a day. The release is controlled by negative feedback, which means that an elevated concentration of GH in the circulation inhibits a further release pulse [9]. Additional pulses can be initiated by external stimuli such as sleep or exercise [10, 11].

Growth hormone is pharmaceutically used to medicate dwarfism as well as growth hormone deficiency in adults [12]. Recombinant human growth hormone (rhGH) is supposed to be widespread as performance enhancing agent because of its lipolytic and anabolic effects [13], although the desired anabolic effects were not confirmed in healthy men and studies examining the performance enhancing effects are controversial [14-16]. Growth hormone is prohibited in sports by the World Anti-Doping Agency (WADA) [11] and two different approaches are available to detect doping with rhGH. The method by Strasburger measures the ratio of the main 22 kDa isoform and other endogenous isoforms and fragments. In this procedure, one sample is analyzed with two different immunoassays. The antibody of the first assay binds the main 22 kDa isoform only and the second assay predominantly detects non-22 kDa variants. The ratio of the assays increases with the abuse of recombinant growth hormone [17]. Another approach is an indirect method from Sönksen and co-workers taking IGF-I and procollagen type III into account [18, 19]. This method enables longer detection times but is still in development.

A new direct method is presented here, which also utilizes the heterogeneity of endogenous growth hormone and the homogeneity of the recombinant analog. It is a proteomics-based approach and uses two-dimensional gel electrophoresis (2D-PAGE) to separate and detect different isoforms. Growth hormone is isolated from human plasma by immunoaffinity purification and narrow range IPG strips are used to separate isoforms of similar molecular weights. After SDS-PAGE, visualization is accomplished by immunoblotting and chemiluminescent detection.

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 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 IPG strips (18 cm, pH 4.7-5.9) 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) 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 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 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. GH in plasma is stable for one week at room temperature or 4°C and for at least 100 days at -20°C [20-22]. 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). To examine the exercise-induced release of GH, a 30 min anaerobic running exercise was performed (mean heart rate: 180 beats/min) with two volunteers (1 male, 34 years, 1 female, 26 years). Samples were taken prior to as well as 0, 10, 20, 30 and 90 min after exercise. Additionally, blood lactate was measured prior to and immediately after exercise.

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: 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.

Isolation of growth hormone from plasma: 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. The primary antibody was diluted 1:2000 in blocking solution, 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.

Pituitary samples and Coomassie Blue staining: For pituitary samples, 5 µL of the extract and 5 µg of lactogen were added to 250 µL of MCSS. The reduction, alkylation and 2D-PAGE were carried out as for the plasma samples. After electrophoresis, gels were stained with Coomassie Blue.

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.

Method validation: The method was validated according to parameters used for mass spectrometry based methods due to its analytical application, and specificity, linearity, limit of detection (LOD), precision and recovery were determined. For linearity, LOD and recovery measurements, horse serum was used as matrix to avoid the presence of endogenous human GH. For specificity measurements, ten plasma samples from ten different volunteers were

prepared to probe for interfering compounds. The calibration curve was prepared by addition of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ng of recombinant GH (Genotropin^{TM}) to 1 mL of horse serum each. The limit of detection (LOD) was calculated from the calibration curve. Additionally, a "visual" limit of detection was determined. A total of 0.25 ng of rhGH was added to six samples of horse serum. For determination of the precision, six identical samples were prepared and the precision was calculated from normalized spot volumes (NSV). The recovery of the immunoaffinity isolation was tested by addition of 2 ng of rhGH to six aliquots of one horse serum sample. In comparison, six aliquots were fortified with 2 ng of rhGH after immunoaffinity isolation. An amount of 20 ng of internal standard was added to each sample after GH isolation.

Results and Discussion

Figure 1 shows Coomassie Blue stained gels of pituitary and recombinant growth hormone. Figure 1 a demonstrates the separation of the growth hormone isoforms from a pituitary extract and Figure 1 b shows a single spot of recombinant growth hormone. Spots **a** and **b** from each picture represent the internal standard. The encircled spots were shown to contain growth hormone and several modified variants were identified by mass spectrometry experiments. Spot **1** as the most abundant GH spot represents the unmodified 22 kDa isoform, while spot **3** was shown to be phosphorylated and spot **2** contains the 20 kDa splice variant [2, 23]. Spot **4,** which has a higher molecular weight of approximately 24 kDa, may be glycosylated which was suggested earlier [3].

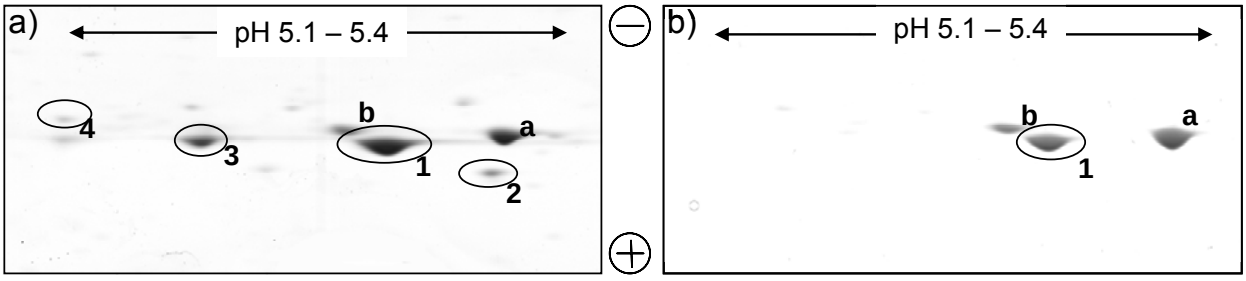

Figure 1 2D-PAGE and Coomassie Blue staining of a) pituitary extract and b) recombinant growth hormone. Encirled spots (1-4) contain growth hormone, spots a and b represent the internal standard

Figure 2 a shows the growth hormone containing spots that were detected in plasma samples (spots **1**-**4**) and the internal standard (spots **a** and **b**). The internal standard, human placental lactogen, has a similar amino acid sequence (sequence homology 86%) and also a similar isoelectric point as growth hormone. Therefore, it can be detected in the pH range of the

employed IPG strips, and the anti-GH antibody is able to bind to the added protein. Placental lactogen is released by placental cells during pregnancy only and is usually not found in the circulation. From the two spots of the internal standard visible on the blot, the more basic and intensive one was used to normalize the GH spot volumes.

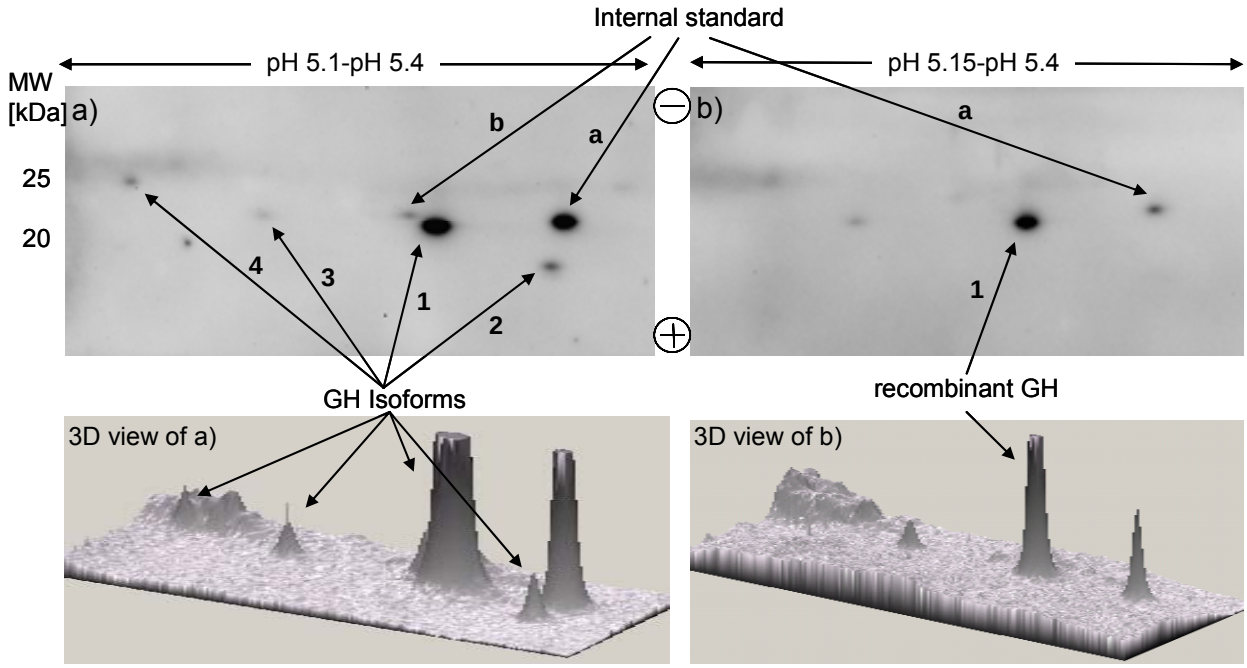

Figure 2 Isolation of growth hormone from plasma, 2D-PAGE and immunoblot. a) human endogenous growth hormone, b) human recombinant growth hormone. Spots 1-4 represent growth hormone variants, spots a and b represent the internal standard.

Validation: Figure 3 shows samples from 8 different volunteers from the specificity measurement. The different concentration of growth hormone results from the pulsatile release from the pituitary which is controlled by negative feedback (6-12 pulses/day) and leads to the detection of different numbers of isoforms. While GH-amounts in sample **4** were below the LOD, samples **1**, **3** and **7** showed one GH spot, representing the unmodified 22 kDa variant. Other samples show two (**5** and **6**) or even four endogenous isoforms of GH (**2** and **8**). Besides the different number of isoforms, no additional signals due to unspecific binding of the antibody were detected that would interfere with the analysis. The equidistant calibration curve ranging from 0.5 – 3.0 ng of rhGH was shown to be linear according to Mandel [24] and the limit of detection calculated from the calibration curve was 0.3 ng. Additionally, a "visual" limit of detection, which was the lowest amount to be detected in six out of six prepared samples, was found to be 0.25 ng. The method showed an acceptable precision of 14 % for the normalized spot volumes, although the absolute spot volumes varied

considerably indicating the necessity of the internal standard. The recovery of the immunoaffinity isolation was 30 %.

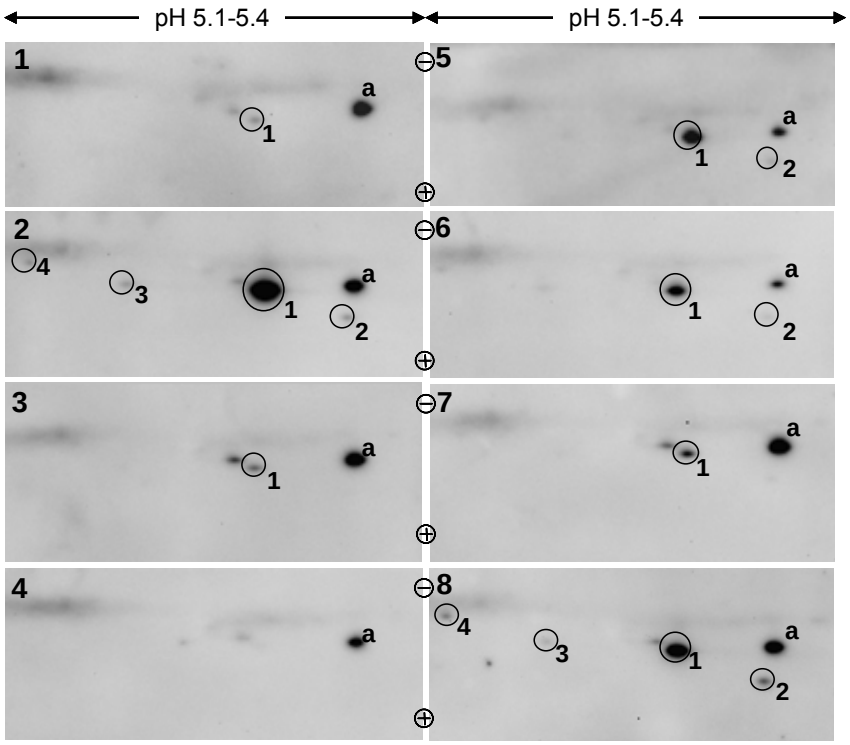

Figure 3 Specificity measurements, Spots 1-4 represent growth hormone variants, spots a and b represent the internal standard.

The validation of such an antibody- and 2D-PAGE-based method with the determination of those analytical parameters is not very common. However, all parameters were successfully specified and especially the good precision and high specificity confirmed the usefulness and robustness of this procedure as analytical method.

Samples from a reference group containing endogenous growth hormone: Reference values for endogenous growth hormone isoforms were required and 33 samples from healthy volunteers were prepared and analyzed. The results of the detected isoforms are shown in Figure 4 (**1**-**33**). The bars represent the normalized spot volumes of spots **1**-**4** as assigned in Figure 2. Different isoform patterns were detected as also shown in the specificity samples. In low intensity samples only the unmodified 22 kDa spot is visible. The second isoform, which appears in more concentrated samples, is the 20 kDa splice variant. Samples with higher amounts of growth hormone show four isoforms. Samples of low concentration that show only the main 22 kDa isoform cannot be distinguished from a low abundant sample containing recombinant growth hormone as the only detectable spot will be identical for both

kinds of samples. After analysis of samples from patients that need to inject recombinant growth hormone, a discrimination limit will be defined. This limit will specify a normalized spot volume of the unmodified growth hormone spot above which more than one endogenous isoform needs to be detected in a normal sample containing endogenous growth hormone. Samples showing only the unmodified 22 kDa spot and a higher value than this discrimination limit will be evaluated as suspicious sample containing recombinant growth hormone.

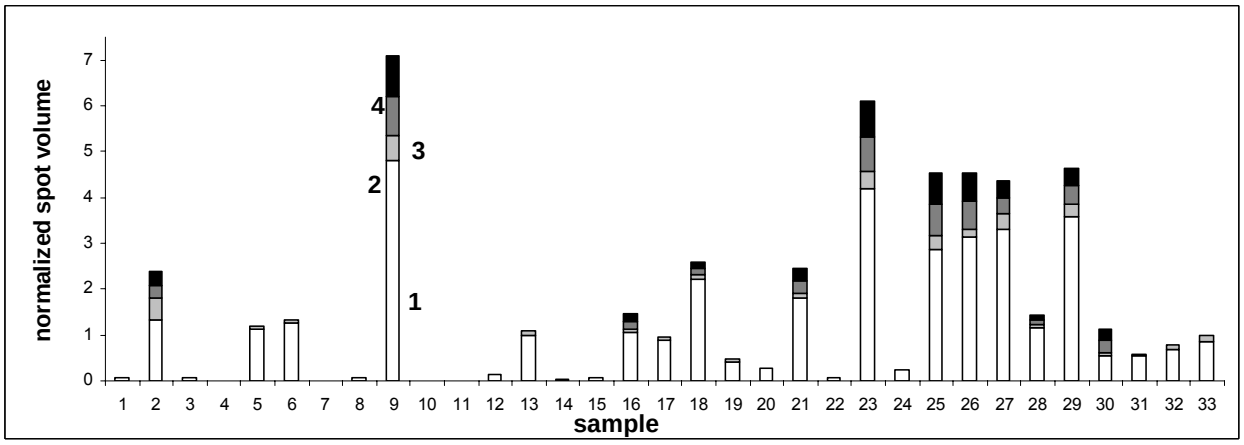

Figure 4 samples from a reference population containing human endogenous growth hormone. Bars 1-4 represent normalized spot volumes of spots 1-4 from Figure 2

Samples from an exercise test:

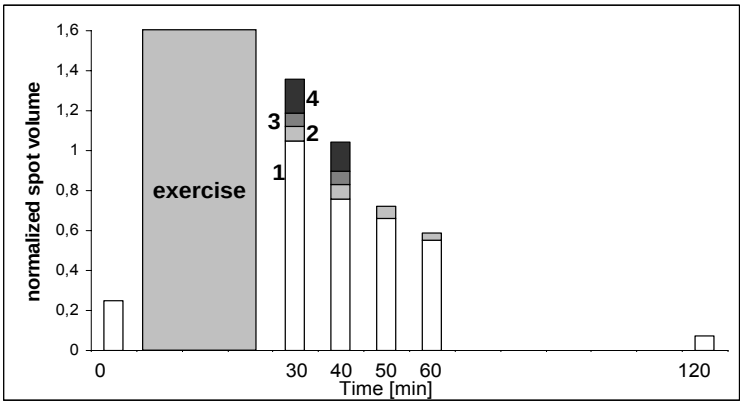

Figure 5 samples from an exercise test. Bars 1-4 represent normalized spot volumes of spots 1-4 from Figure 2. An amount of 0.25 mL of plasma was used for the analysis.

Exercise is known to stimulate GH release which is connected to the increase in blood lactate during anaerobic exercise [10]. A standardized exercise test was carried out to assure that an exercise induced release of growth hormone can be detected as an increase in all endogenous isoforms as it was shown for immunoassays before [25]. Lactate levels were elevated from 1.0 mmol/L prior to exercise, to 7.9 mmol/L after exercise. Figure 5 shows that the GH

concentration in the circulation was increased immediately after exercise and that the abundance of all detectable isoforms was elevated. Prior to exercise only one spot was visible due to low circulating amounts of GH while after exercise, all four isoforms were present in measurable concentrations. Therefore, the release of endogenous growth hormone in response to exercise can be measured as an elevation in all detectable endogenous isoforms as for regular release pulses.

Concluding remarks

Growth hormone is supposedly widely abused by athletes for its anabolic and lipolytic as well as growth promoting effects. The presented method is capable of discriminating endogenous and recombinant growth hormone in plasma or serum samples if the concentration is high enough to detect endogenous isoforms. Analysis of samples from growth hormone deficiency patients and a larger reference population will allow the statistical determination of a discrimination limit that enables the application of this method as anti-doping control method to detect the abuse of recombinant human growth hormone.

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