In: W Schänzer, H Geyer, A Gotzmann, U Mareck (eds.) Recent Advances In Doping Analysis (13). Sport und Buch Strauß - Köln 2005

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Detection of hGH doping by means of isoform selective immunoassays-3

Isoform ratio of hGH in serum and new approach to automate array detection of proteins by means of flowcytometry

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The major isoform of the human growth hormone is a 22KDa single chain peptide (22K) secreted by the pituitary gland, which consists of 191 amino acid residues with two disulfide bonds. The translational loss of 15 amino acids (32 to 46) by active splicing results in the formation of another minor isoform (20KDa-hGH:20K). The chemical structure of recombinant GH is exactly same as that of pituitary 22K. Therefore, elevated concentrations of total hGH and/or 22K isoform are not sufficient as scientific evidence of hGH doping. The aim of this study was to construct methods (enzyme-linked immunosorbent assay: ELISA) for measuring levels of 22K and 20K in biological samples and to apply this system in the detection of hGH doping. The ELISA showed not any significant cross-reaction between isoforms or with other structurally related peptide hormones demonstrating the marked specificity of these assays. The limit-of-quantitation for both isoforms was 5.0 pg/ml respectively, and the methods were linear up to 3,000 pg/ml. The respective recoveries for 22K/20K at 10.0/100.0 pg/ml were 97.0/96.7(%). and their expanded uncertainty U(P) were 0.8P and 1.8P at coverage factor: k=2. For integration of the methods into screening procedure, we have been trying to measure 20K and 22K simultaneously by means of a flowcytometer. Each antibody was immobilized on microbeads, and multiplexed immunoassays were

achieved with the mixed antibody-coated microbeads. Each single target was quantified after sorting the microbeads by flowcytometer. Using the multiplexed assay, hGH isoforms were detected simultaneously in one test with good measurement traceability to microplate ELISAs. The results of the multiplexed detection are also referred to in this report.

MATERIALS AND CRITICAL REAGENTS:

Recombinant 22K was Somatropin (5.33mg from Pfeizer, Japan) and was calibrated against WHO98/574 from NIBSC Lot.050413) before being put into service. Recombinant 20K was synthesized according to the procedure published in literature by Uchida et.al.[1] Anti-22K mouse monoclonal antibody with the clone number A36030047P containing 0.1% NaN_3 was purchased from Biospacific (CA, USA), and anti-20K mouse monoclonal antibody D05 was re-cloned in the laboratory. Affinity purified anti-human GH goat polyclonal antibody 421067 was purchased from Techne Corporation (Minneapolis, USA). AMDEX® streptavidin-HRP (Amersham Biosciences, Catalog No.RPN4401V), Mouse Serum (Chemicon, Catalog No.S25-10ML), TMBZ: 3,3',5,5'-tetramethylbenzidine (TMBZ); TMZ Microwell Peroxidase Substrate “2-Component System” (KPL: Kirkegaard & Perry Laboratories, Inc., Catalog No.50-76-00), HBR: HBR 1–Purified Heterophilic blocking reagent-1 (Scantibodies Lab., Santee, California, USA, Catalog No. 3KC534), FluoReporter: sulfosuccinimidyl ester Biotin-XX Protein Labeling Kit (Molecular Probes, OR, USA, Catalog No.F-6347), Blocking agent: Block Ace (Dainihon Pharmaceuticals, Catalog No. UK-B25), Protein-A: Affi-Gel Protein-A MAPS II Kit (BioRad, Catalog No. 153-6159, CA, USA), Microplate: Nunc-Immuno Module (F8Maxisorp, Catalog No.469949, Nalge Nunc International KK, Hongo, Tokyo).

Reagents prepared in house: a) Assay and sample dilution buffer: 1.37M-NaCl 100mM-phosphoric acid containing 2% BSA, 10mg/l HBR, 0.1% Tween20 and 0.1% mouse serum pH=7.4. b) Calibration blank: 2%BSA in 1.37M-NaCl, 100mM-phosphoric acid

pH=7.4. c) Dilution buffer for biotin-labeled anti-human GH goat polyclonal antibody solution: 0.5%BSA, 0.05%Milk Casein, 0.5% mouse serum and 0.1%-Tween20 in 0.137M-NaCl 10mM-phosphoric acid buffer pH=7.4. d) Dilution buffer for streptavidin conjugated to HRP: 10%-BlockAce in 10mM-PBS pH=7.4. The conjugate was diluted to 1:5000 as suggested by the supplier's instruction. e) Reaction terminator: 1M-phosphoric acid. f) Microplate washer liquid: 10mM-PBS, 0.05%Tween 20, pH=7.4.

ELISA assayplates: Isoform selective antibodies (5 micro g of A3603004P or 10 micro g of D05 per well) were aliquoted to each well, and the microplate was stood for 48 hrs at 4°C. The excess antibody solution was then removed by 3 cycles of washing steps with microplate washer and 25%-BlockAce aqueous solution, and the final washing step was stopped with microplate washer. Then, the assay plates were dried and stabilized at least for 2 weeks by storing under vacuum at 4°C before put into service.

Preparing biotin-labeled antibody: Biotinyl antibody was prepared according to the supplier's instruction of "FluoReporter Biotin-XX Protein Labeling Kit". The resultant reaction mixture was used as the secondary antibody.

Human samples: MBC gathered plasma samples from healthy adults in the laboratory and some athletes as a part of the routine blood testing program. Serum samples of elite athletic athlete were collected by Japan Associations of Athletic Federations (JAAF) in cooperation with MBC as a part of the athletes' health check program. The subjects included JAAF-registered top middle- or long-distance runners including 46 male adults, 36 female adults and 16 adolescent males.

Analytical Instruments: Immunoplate reader:(Sunrise®, TECAN Trading AG, Geneva), Flowcytemeter Multiplexed Array Detection System: Luminex® 100 Complete System from Luminex Corporation (Austin, TX, USA), Surface Plasmon Resonance analyzer: SPR3000 BIA-core (Pharmacia Biosensor, Tokyo, JAPAN).

METHODS

Each assayplate was washed twice with assay buffer prior to use, and 75 micro l of assay buffer were added. Then, either and 25 micro l of calibrator, controls or serum samples were evenly applied to each well. After being shaken for 10 seconds, the assayplate was incubated for 20 hrs at 4°C and washed with 5 cycles of assay buffer. The second reaction with the captured GH isoform was achieved by incubation with 100 micro l of biotinyl-anti-hGH goat polyclonal antibody 421067 for 2 hours at room temperature. After washing cycles, 100 micro l of streptavidin labeled HRP-linked anti-goat IgG antibody in conjugate buffer was added to form the Biotin-Avidin complex by incubation for 1 hour at room temperature. In the final step of the analyses, 100 micro l of TMBZ peroxidase substrate solution was added to each well, and the reaction mixture was kept for 20 minutes at room temperature under darkness. The reaction was terminated with 0.1ml of 0.1M-sulfuric acid, and absorbance at 450 nm was measured with the reference at 620 nm. Mathematical calculations were done with SOFT max® PRO software Version3.1.2 (Molecular Devices Corp., CA, USA), and StatFlex Ver.4.2.rel.3 (Artech, Osaka, Japan) was used for further statistical evaluation.

RESULTS AND DISCUSSION

The results showed D05 to be the sole antibody, which could specifically react with 20K. D05 does have high affinity against 20K in the liquid phase [2], but the reactivity of D05-20K complex with the second antibody is poor when D05 is immobilized on a solid surface, according to the results of the evaluation study by surface plasmon resonance (SPR) or by ELISA on a microplate. Such a problem is probably caused by the orientation of D05 Ig-G or Ab-Ag complex on a solid surface, which may affect the binding of the secondary antibody. We found that the affinity of another antibody D14-HRP against 20K in sandwich ELISA was much less than that against 22K, but 421067 showed marked sensitivity both to 22K and 20K. A36030047P recognizes any part of the amino acid sequence between 32 to 46 and was found

to be the most suitable anti-22K antibody for sandwich ELISA for 22K.

Sensitivity and linearity: Both for 20K and 22K ELISAs, LOQ was 5.0 pg/ml. The assay ranges were set over 5.0 to 1,000 pg/ml for 20K and 10.0 to 2,000 pg/ml for 22K, because natural abundance of 22K and 20K in humans is roughly 10:1. Methods were linear over the range studied with the CV=1 to 7.8 %, and serum samples with isoform concentration out of the upper range could be re-analyzed after a maximum of 32 times of dilution with an assay buffer. Samples with concentration of the target out of the assay range were retested after sample dilution.

Recovery: Normal serum samples spiked with three levels of GH isoform were analyzed and the recoveries were calculated. Analysis was done in triplicate. The overall recovery of GH within the assay range was 78-100% and found to be close between isoforms, so the isoform ratio was calculated without any correction.

Uncertainty of measurements: For both of the ELISAs, the standard uncertainties and relative standard uncertainties, such as purity of reference standards, metrological errors etc. were regarded as negligible. The biggest uncertainty source originated from the back calculation error generated through curve fitting of the equation formula for the calibration. The expanded uncertainty: U(P) at coverage factor k=2 (confidence level approximately 95.4%) for 22K, 20K and 22K/20K were 0.8P, 1.8P and 2.2P respectively.

Human samples

1) Normal adults (recreational sports group)

A sex dependent difference of total GH concentration was observed, and the mean value in females was higher than that in males. As shown in table-1, however, changes of isoform levels in serum were more or less parallel and no sex dependent difference of the ratio was observed. There was also an age dependent decrease of GH excretion particularly in females. As the age-dependent decreases of isoforms occur in parallel, however, the variation does not cause in any adverse effect on the isoform ratio. 22K/20K ratios 10.5 +/- 7.2 and 10.6 +/- 5.0

for males and females, respectively, were found. (Data not shown)

Table-1 Results of isoform assays on healthy normal adults

Male	n	Mean	SD	CV	Min	Max
22K+20K	24	628	794	127	45	2882
22K	25	550	746	136	31	2771
20K	24	58	57	97	8	218
22K/20K	24	10.5	7.2	68.7	0.9	26.8
Female	**n**	**Mean**	**SD**	**CV**	**Min**	**Max**
22K+20K	13	1295	1347	104	166	4387
22K	13	1174	1234	105	151	3979
20K	13	122	128	105	15	408
22K/20K	13	10.6	5.0	46.9	4.2	20.6

Unit: Concentration pg/ml, 22K/20K Ratio g/g

2) Elite athletic athletes (middle- and long-distance runners), Figure-1

In 10 male athletes of this subject group, the 20K levels were not measurable (<LOQ: 5pg/ml). Such naturally suppressed levels of 20K were easily distinguished from GH doping as the total GH and 22K were as extremely low as about 100 pg/ml. 20K concentrations in female athletes were measurable in all cases. A sex dependent difference of concentrations of GH was also the case for elite athletic athletes. Naturally elevated levels of the GH concentrations in female athletic athletes were observed more extensively than that observed in recreational female adults. Even though the difference of GH concentrations between females and males of this group was about 5:1, the 22K/20K fell within the same range, and no significant sex dependent difference was found. The sex dependent difference of GH concentrations was successfully corrected by monitoring the 22K/20K. Calculated ranges of 22K/20K in males and females were 11.7 +/- 7.2 and 10.4 +/- 6.0, respectively. Overall results of our study showed that 22K and 20K isoform does have diurnal, sex- and age-dependent variations,

which are already well known for total GH. Regardless of the large variations, however, the isoform ratio is fairly stable since concentrations of GH isoform concomitantly change with other GH isoforms. For all subject groups studied, the 22K/20K was roughly about 10:1, and physiologic variations of the GH secretion could be successfully compensated by monitoring concentration of 20K as the endogenous reference hormone (ERH).

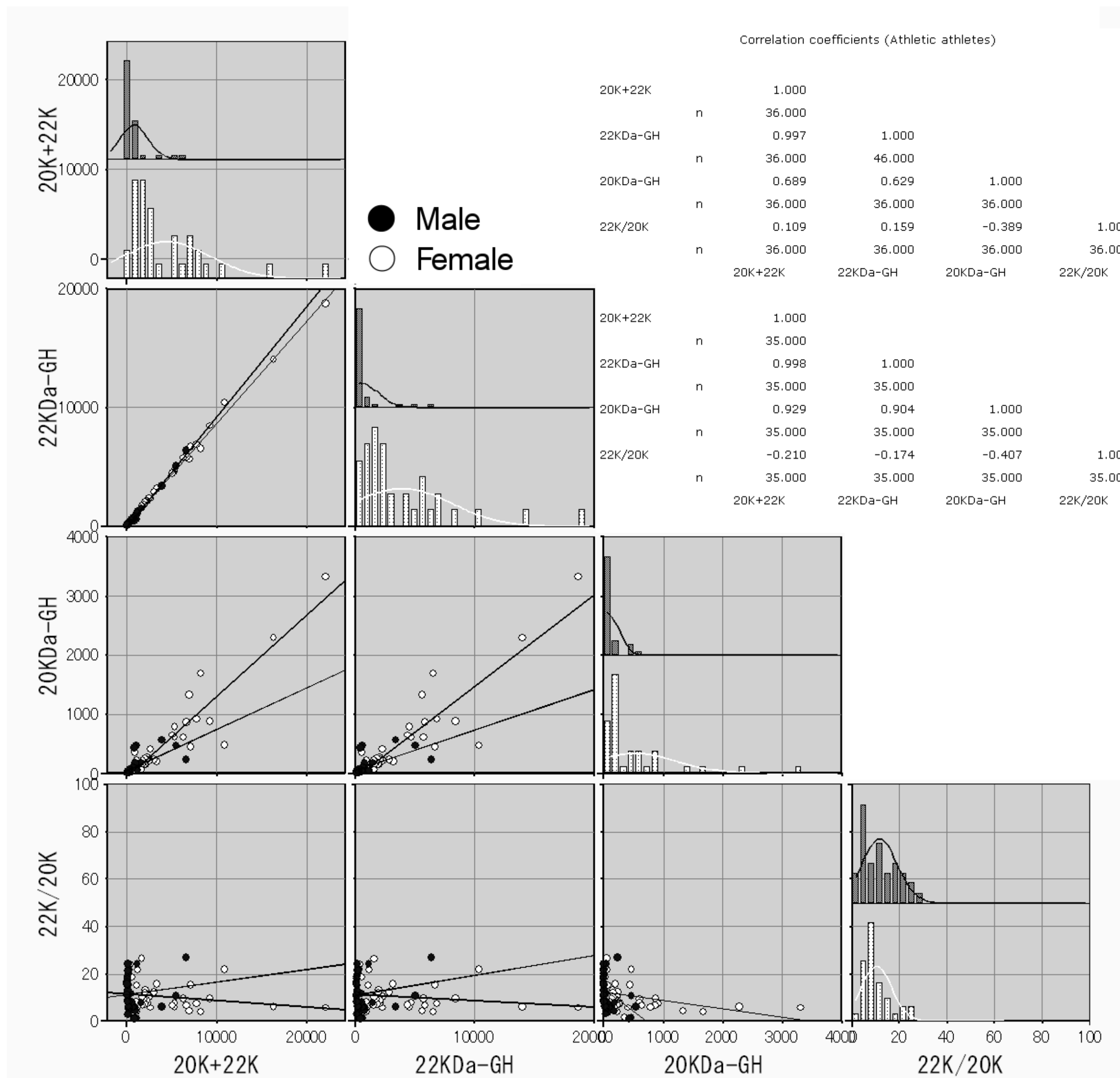

Figure-1 Distributions of serum levels of GH parameters and their 2-D correlations in elite athletic athletes. *Data were divided by sex. (Unit: Conc. pg/ml, 22K/20K Ratio g/g)*

Interpretation of analytical results

The potential application of our study is the direct detection of elevated levels of 22K in

serum after injection of rhGH by means of simultaneous monitoring of 20K as an individual ERH. Our results show that the isoform ratio in non treated individuals is fairly constant, and intra-individual isoform ratios fell within narrow range. It was observed in our previous studies [3] that pituitary secretion of GH in humans would be suppressed when rhGH was injected due to reciprocal negative feedback between isoforms. The concentration of 22K reached a maximum and that of 20K were suppressed in a few hours after the injections. In all three of the subjects presented at 22nd Cologne Workshop, the isoform ratios increased 3 hours after the administration, and remained at higher levels until 72 hours after the injections. The isoform ratio could not always be estimated when the concentrations of 20K were completely suppressed. In such cases, ratios of 22K concentrations of the samples to LOD of 20K(5pg/ml) were calculated for result evaluation. Even in such a situation, the ratio indicated the abuse of rhGH between 3 to 72hours after the single dose GH administrations. Although the durability of elevated 22K/20K was fairly short, the use of the parameter was considered to be important for doping control, as the isoform ratio did not show any significant sex dependent difference or age-dependent decrease which are well known for GH.[4,5] These observations suggested that the increase in the ratio of 22K/20K in serum would directly reflect both the pharmacokinetics of exogenously administered 22K and the suppression of endogenous GH secretion.

Further consideration of the method integration into testing system [6, 7]

In this procedure, primary anti-hGH isoform antibody was immobilized on polystyrene micro-beads, which giving a unique fluorescence ("Spectral Address"), and multiple immunoassays were performed in a medium suspended with a mixture of the antibody coated micro-beads. At the final step in the analysis, individual microbead was sorted in the liquid sheath flow using a flowcytometer according to each spectral address, and then intensity of accumulated fluorescence was measured for each hGH isoforms. Thus, those multiplexed immunoassays could be achieved simultaneously in one tube with a single serum aliquot at

levels of about few pg/ml. According to the results of our studies on Luminex® (figure 2 and 3) the method was confirmed to be traceable to and more sensitive than sandwich ELISA.

In summary, our approach to the use of state-of-the-art multiplexed immunoassays for target biopolymers, such as hGH isoforms, indirect born markers, and other peptide hormones by means of flowcytometer is considered to be a possible tool for the screening of peptides.

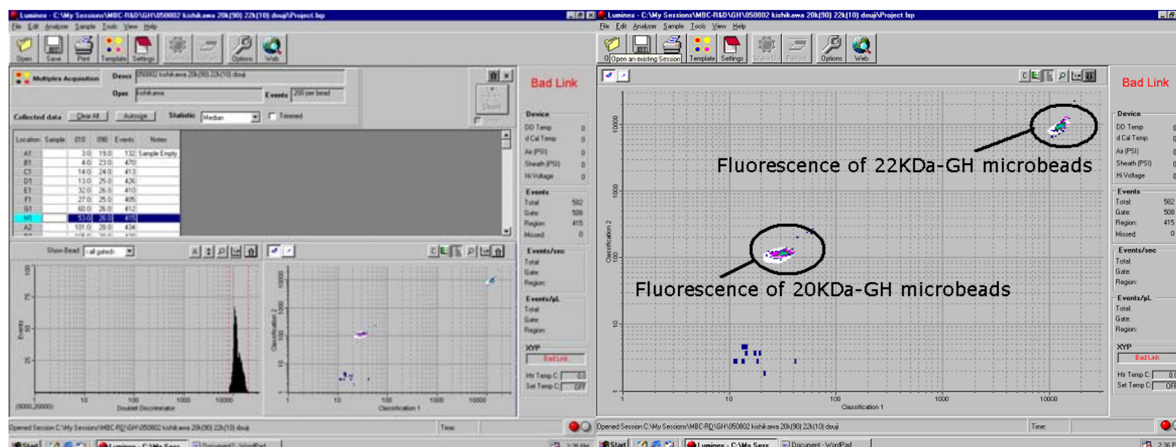

Figure-2 Typical view of control panel of flowcytemeter multiplexed array detection system *Luminex*®; showing isolation of antibody microbeads and accumulation of fluorescence divided by hGH isoforms.

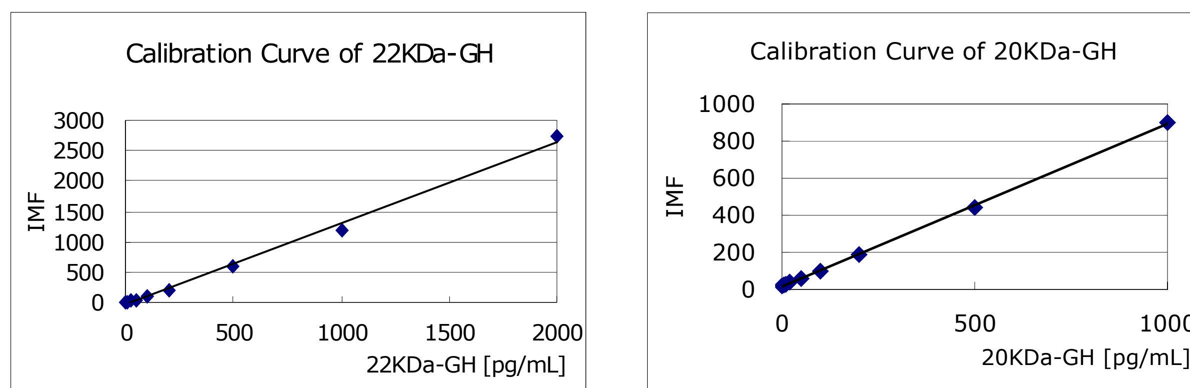

Figure-3 Linearity of multiplexed hGH isoform assay. (fluorescence vs concentration)

ACKNOWLEDGMENT

This research was partly supported by the grant from WADA (IMIM/Barcelona – MBC/Tokyo joint research project for "Chip technology for the detection of growth hormone abuse") and

the Society for Growth Sciences Japan. Thanks are also due to cooperation by Dr. F. Yamasawa and JAAF for collecting athletic athletes' samples, useful suggestion by prof. Minoru Irie and initiative in GH human studies done in 2000 by Dr. Takashi Kawahara.

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