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Validation of Differential Immunoassays of hGH Isoforms; KIT 1 and 2

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Introduction

Growth hormone (GH) is a peptide (191 amino acids) that stimulates growth and cell reproduction in humans and animals, and it is synthesized, stored, and secreted by the anterior pituitary gland. Pituitary GH (pit GH) is secreted in several different molecular isoforms; 22 kDa (approx 40-45%), 20 kDa (7-8%), dimers of both of these (20-30%), and smaller percentages of modified and fragmented GH. Recombinant GH (rec GH) is composed solely of the 22 kDa isoform. Drugs containing the growth hormone can be abused by athletes and therefore it has been added to the list of banned substances published by the World Anti-Doping Agency [1].

New method must be validated prior its implementation to an anti-doping laboratory. However, there are no precise guidelines from WADA how to perform appropriate validation of immunoassays so each laboratory must face this problem on its own. Here we present our approach to validate the Differential Immunoassays of hGH Isoforms; KIT 1 and 2. Moreover, our preliminary data suggest that the results of hGH anti-doping tests may be masked by an unknown agent present in sera of pregnant women.

Methods

The method used to determine hGH concentrations was ”hGH LIA differential Immunoassays of hGH isoforms KIT 1 and 2” produced by CMZ-Assay. Each of these kits is composed of two different 2-step sandwich immunoassays: Rec and Pit. Antibodies utilized in the Rec assays preferentially recognize the monomeric 22 kDa isoform of hGH, while antibodies utilized in Pit assays bind its several different isoforms [2].

Results and discussion

Measurements of uncertainties (MU) of the methods were derived based on 5 series of measurements done by two persons. In each series, 14 measurements were done using the

same control serum sampled from one volunteer (results are shown in Table 1), and measurements of control sera K1 and K2 (not shown).

Table 1. Standard uncertainties of control sample.

	KIT 1 PIT ng/ml					KIT 1 REC ng/ml				
	Day1	Day2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Mean	8.94	9.44	8.87	9.8	8.93	8.51	7.75	7.89	7.98	8.05
%CV	5.94	4.65	3.84	6.59	4.04	5.54	4.19	5.23	5.37	4.39
	KIT 2 PIT ng/ml					KIT 2 REC ng/ml				
	Day1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Mean	9.28	8.9	7.45	7.54	8.31	7.93	7.89	7.57	7.39	7.4
%CV	2.86	5.06	3.82	4.19	3.59	6.5	4.31	6.2	3.55	3.26

To calculate the combined uncertainty of the rec/pit ratio we took into account two sources of uncertainties: the standard uncertainty and pipetting errors. The combined uncertainty of the rec/pit ratio for KIT1 and KIT2 was 9% and 11%, respectively. We calculated the expanded uncertainty as follows: the combined uncertainty was multiplied by the k factor =2 (t-student distribution, the level of confidence 95%). We obtained the expanded uncertainty 21.8% for KIT1 and 19.6% for KIT 2. The limit of quantification was determined by measurements of a sheep serum (serum without the human growth hormone) and calculated as a mean value of the results plus standard deviation of obtained values multiplied by ten. Results for the KIT 1 were: rec 0.006 ng/ml; pit 0.04 ng/ml and for the KIT 2 were: 0.008 ng/ml and 0.02 ng/ml.

To determine physiological states which can affect the result of the hGH anti-doping tests we performed literature data mining. We found that the placental growth hormone variant hGH-V shows cross-reactivity with antibodies used in the KITs [2]. The human placental GH (hGH-V) is a variant of the pituitary hGH, and it is synthesized and secreted by syncytiotrophoblasts during pregnancy. The hGH-V is present in human body only during pregnancy from 7 GW (gestational week) [3]. It differs from hGH by only 13 amino acid residues [3]. In the paper by Bidlingmaier et al., (2009) authors tested sheep serum spiked with hGH-V and showed cross-reactivity from 14% up to 28% with different assays, and rec/pit ratios for sheep serum spiked with 10 µg/L hGH-V were 0.51 (rec2/pit2) and 0.60 (rec1/pit1). Since no samples collected from pregnant women have been analyzed so far, we decided to check whether gestation affects results of the hGH differential immunoassays; KIT 1 and 2.

The results of analyzes of twenty sera taken from pregnant women (GW from 7 to 37) are shown in Table 2.

Table 2. Analysis towards hGH rec and pit content of sera taken from pregnant women.

		KIT 1			KIT 2		
	GW	**REC ng/ml**	**PITng/ml**	**REC/PIT**	**REC ng/ml**	**PIT ng/ml**	**REC/PIT**
1	7	1.22	1.25	**0.98**	1.41	1.53	**0.92**
2	8	0.91	1.26	**0.72**	1.22	1.26	**0.97**
3	8	13.92	18.46	**0.75**	15.64	21.89	**0.71**
4	9	2.9	3.93	**0.74**	3.13	6.03	**0.52**
5	9	4.75	6.34	**0.75**	4.94	8.11	**0.61**
6	14	0.15	0.81	**0.19**	0.56	0.81	**0.69**
7	15	1.32	8.49	**0.16**	7.1	11.4	**0.62**
8	19	0.38	5.5	**0.07**	3.83	6.67	**0.57**
9	21	0.09	1.44	**0.06**	1	1.13	**0.88**
10	24	0.06	2.37	**0.03**	1.8	2.84	**0.63**
11	25	0.05	1.44	**0.03**	1.47	1.52	**0.97**
12	26	0.04	5.01	**0.01**	2.03	5.71	**0.36**
13	27	0.04	3.52	**0.01**	1.8	3.56	**0.51**
14	27	0.03	1.3	**0.02**	1.03	1.61	**0.64**
15	27	0.05	1.18	**0.04**	1.6	1.65	**0.97**
16	28	0.04	2.19	**0.02**	1.11	1.7	**0.65**
17	31	0.03	4.26	**0.01**	1.47	3.54	**0.42**
18	34	0.05	2.52	**0.02**	1.5	1.98	**0.76**
19	37	0.05	2.08	**0.02**	1.46	2.28	**0.64**
20	37	0.05	2.26	**0.02**	1.84	2.63	**0.70**

We observed similar rec/pit ratios for samples taken from women in GW 7 – GW 9 for KIT 1 and KIT 2 as expected. Surprisingly, from GW 14 we observed different levels of rec hGH depending on KITs used. KIT 1 showed much lower rec hGH levels and a lower rec/pit ratio as compared to KIT 2. The cross-reactivity of the rec assays reported by Bidlingmaier et al., (2009)[2] were similar (16,1% KIT 1 and 14,1% KIT 2), thus it possible that the antibody used in the rec assay of KIT 1 may preferentially bind to other protein(s) than hGH and hGH-V, and present in sera of pregnant women. This putative protein is probably not recognized by a secondary antibody, hence the result of the assay is close to zero. The other explanation may be that the recombinant hGH-V used in the study of Bidlingmaier et al. (2009) [2] has different affinity for antibodies utilized in the KITs 1 and 2 than endogenous one present in serum taken from pregnant women.

Conclusions and perspectives

The measurements of uncertainties were estimated at the level of approximately 20% for both KITs. However, our long-term validation is in progress allowing us therefore to monitor the methods and to recalculate our uncertainty in measurements in future.

It seems that pregnancy may affect results of anti-doping analysis toward abuse of the recombinant hGH. It is conceivable that sera of pregnant women may contain protein(s) which binds to an antibody used in the rec assay of KIT 1 with high affinity and therefore can mask the recombinant hGH. Hence, this putative protein (if is not harmful for human) can be used as a masking agent by athletes abusing the recombinant hGH. On the other hand, it can be secreted in certain pathological conditions (e.g. cancer) therefore identification of it seems to be interesting future challenge.

References

[1] WADA 2009 Prohibited List; http://www.wada-ama.org/rtecontent/document/2009_Prohibited_List_ENG_Final_20_Sept_08.pdf

[2] Bidlingmaier, M., Suhr, J., Ernst, A., Wu, Z., Keller, A., Strasburger, C. J. and Bergmann, A. (2009). High-sensitivity chemiluminescence immunoassays for detection of growth hormone doping in sports. *Clin Chem.* **55,** 445-53.

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