YANG, Sheng; HE, Chunji; DONG, Ying; WANG, Shan; XU, Youxuan; WU, Moutian; JING, Zimeng; WU Qinyong; ZHU Huijuan; PAN Hui; GONG Fengying; GU Feng

The use of "Kit A" for the detection of rhGH doping

National Anti-Doping Laboratory, China Anti-Doping Agency, Beijing, China, 100029
Department of Endocrinology, Peking Union Medical College Hospital, Beijing, China, 100730

Introduction

As proposed by WADA before the 2008 Beijing Olympic Games, two kits made by CMZ GmbH in Germany were used for detecting doping with rhGH, with Kit B being used for the initial test and Kit A for the second test each having its own reporting ratio threshold (recGH/pitGH) for adverse analytical findings (AAF) as listed below:

	Males	Females
Kit A (second test)	2.17	1.68
Kit B (initial test)	1.64	1.19

Different monoclonal antibodies are used in the test kits as follows: 1B3 pitGH (AK565) and 5D7 (AK566) recGH for the initial assays (Kit B); and 8A9 (AK567) pitGH and 811B (AK568) recGH for the second assays (Kit A). AK569 is used for both kits as the tracer [1].

After the Beijing Olympic Games, some batches of Kit A remained unused. The aim of this study is focused on 1) a population investigation using Kit A; 2) a comparison of the ratios obtained with both Kits A and B, and 3) a comparison of the GH concentrations obtained with both Kit A and another commercial Kit (DPC Immulite).

The serum samples were collected from three groups: Group A: patients without rhGH administration (Acromegaly/Pituitary Gigantism/Pituitary Adenoma), 122 female and 82 male sera were collected by the Peking Union Medical College Hospital (PUMCH); Group B: patients with rhGH administration (Growth Hormone Deficiency (GHD) /Turner Syndromes),

39 female and 30 male sera were obtained from the Beijing Children's Hospital. Group C: Healthy population with 29 female and 30 male sera. In addition, data from an independent data set were also reviewed and compared.

Methods

Following the kit procedures provided, all samples were analyzed both with Kit A for the ratios and with DPC Immulite for GH concentration. Samples of Group C were additionally analyzed with Kit B. Statistics were carried using Excel and SPSS (v.10).

Results

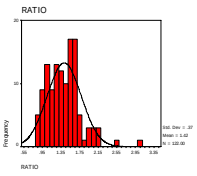
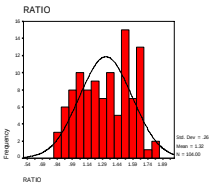
1) Quality Control of Kit A: The quality control data listed below were achieved by three different analysts during the 20 days of measurements. These data confirmed that the deviation on concentrations or ratios were less than 6 %. The same variability was found with Kit B in both our analyses and those published [1].

Table 1 Quality Control Results

Control	Item		SD (n=7)	CV%
Control 1 (negative)	recGH	0.855 ng/ml	0.034	4.0
	pitGH	0.872 ng/ml	0.036	4.1
	Ratio	0.982 < Criteria	0.053	5.4
Control 2 (positive)	recGH	3.526 ng/ml	0.101	2.8
	pitGH	1.312 ng/ml	0.078	5.9
	Ratio	2.692 > Criteria	0.092	3.4

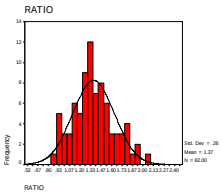
2) Analytical Data of Group A: Data from 122 female sera in Group A, including 18 sera with ratios greater than 1.68, are listed in Table 2A; the ratios obtained from 104 females, excluding the above 18 specimen, are shown in Table 2B; the respective statistical distribution graphs are also shown.

Table 2 Analytical Data of Female Sera in Group A

2A	recGH (ng/ml))	pitGH (ng/ml)	Ratio	DPC GH (ng/ml)	Statistical Distribution
Mean	11.588	8.566	1.418	16.170	
SD	10.057	8.799	0.366	11.146	
Median	7.734	5.915	1.419	11.550	
Min.	2.312	1.370	0.821	3.300	
Max	67.132	81.802	3.052	51.200	
2B	recGH (ng/ml))	pitGH (ng/ml)	Ratio	DPC GH (ng/ml)	Statistical Distribution
Mean	11.108	8.991	1.324	15.965	
SD	9.632	9.129	0.265	11.000	
Median	7.875	5.975	1.333	11.800	
Min.	2.312	1.370	0.821	3.300	
Max	67.132	81.802	1.788	51.200	

The data obtained from the 82 male sera are shown in Table 3. As can be seen, no ratio was greater than 2.17.

Table 3 Analytical Data of Males Sera in Group A

	recGH (ng/ml))	pitGH (ng/ml)	Ratio	DPC GH (ng/ml)	Statistical Distribution
Mean	16.303	13.039	1.373	16.170	
SD	18.207	16.575	0.264	11.146	
Median	7.407	4.644	1.359	12.950	
Min.	1.236	0.946	0.862	1.900	
Max	63.763	63.972	2.034	62.500	

3) Analytical Data of Group B: All patients in this group were treated with rhGH for a period of three weeks to around 12 months (no information about the exact date of the last dose was available). The distribution of ratios of female sera is shown in Figure 1A, together with that of male sera in Figure 1B.

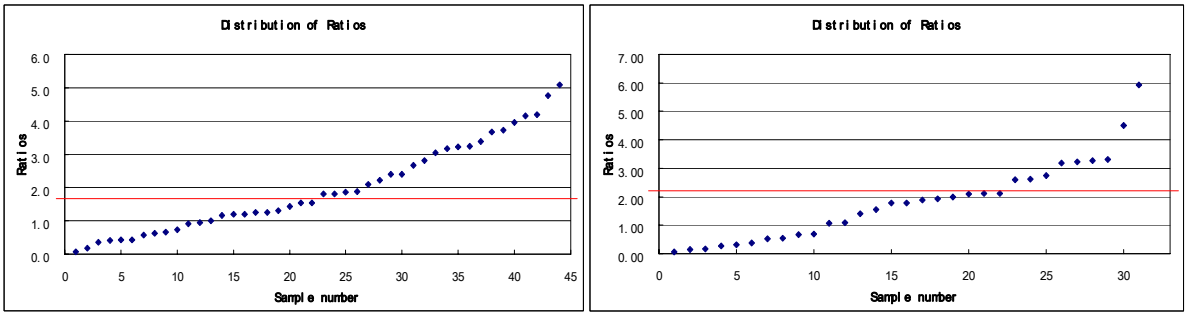


Figure 1 Ratios of Sera of Group B (Figure 1A on the left, Figure 1B on the right)

4) Ratios of Sera of Group C: All ratios obtained directly from Kits A and B with the unadjusted concentrations as well as the statistical results are listed in Table 4.

Table 4 Ratios of Sera in Group C

	Kit A		Kit B	
	F (n=29)	M (n=30)	F (n=29)	M (n=30)
Mean	0.986	0.945	0.569	0.528
SD	0.296	0.369	0.168	0.244
Median	0.987	0.923	0.562	0.494
Min	0.483	0.312	0.246	0.119
Max	1.503	1.741	0.911	1.008
Skewness	-0.144	0.136	1.709	0.126
S.E. of Skewness	-0.433	0.427	0.434	0.427
Kurtosis	-1.036	-0.618	2.376	-1.155
S.E. of Kurtosis	0.845	0.833	0.845	0.833

Discussion

1) Difference of Ratios between Genders: As different criteria for females and males have been proposed by WADA, it is generally accepted that these different thresholds reflect the difference of ratio distribution between the genders. However, based on our careful investigations, either in Group C with both Kits A and B (Table 3) or in Group A with Kit A (Tab 2B) only, no significant difference could be observed in the ratios between genders. A recently published paper [2] declared that for normal males (n = 392), the ratio of 20 kDa-GH to 22 kDa-GH was in a range of 9.5 ± 3.4 %, for normal females (n = 50), the ratio fell into the range of 8.4 ± 4.4 %. These results confirmed that no significant difference in ratios was observed between genders. In other relevant papers [3, 4], hGH concentrations in females are higher than in males of comparable age, and as calculated over 24 hours. Bidlingmaier and co-workers [1] also showed that the ratios did not differ significantly between sexes (rec/pit A, $P = 0.84$; rec/pitB, $P = 0.61$) and the results were independent of either age or absolute hGH concentration ($P > 0.05$). The authors concluded that from the data of 2 independent cohorts of healthy subjects, no sex difference in the ratios was observable.

Careful review of the data from other independent cohorts of healthy subjects from our routine analyses obtained using Kit B, showed no obvious difference in ratios between genders and that the slightly higher ratios of males might be caused solely by the generally lower concentrations in male subjects. Please refer to Table 5.

Table 5 The Ratios with Different Concentrations of pitGH

Item	**Total**		**0.09 ng/ml ≤ pitGH**		**0.05 ≤ pitGH < 0.09 ng/ml**		**pitGH < 0.05 ng/ml**	
	M	F	M	F	M	F	M	F
n	337	133	235	121	46	5	56	7
Mean	0.602	0.562	0.482	0.528	0.585	0.681	1.122	1.057
SD	0.529	0.231	0.205	0.195	0.161	0.114	1.084	0.288
Median	0.518	0.537	0.448	0.511	0.572	0.648	0.800	1.067
Min	0.078	0.097	0.078	0.097	0.299	0.604	0.457	0.586
Max	8.000	1.538	1.168	1.030	0.983	0.882	8.000	1.538

All ratios by gender are listed in Table 6, which did not show any significant difference.

Table 6 The Ratios of Different Populations with Genders

Item	Kit A		Kit B	
	Males	Females	Males	Females
Normal Population*	----	----	166	110
Mean	----	----	0.514	0.538
SD	----	----	0.213	0.198
Median	----	----	0.493	0.526
Min	----	----	0.087	0.097
Max	----	----	1.17	1.03
Normal Population	30	29	30	29
Mean	0.945	0.986	0.528	0.569
SD	0.369	0.296	0.244	0.168
Median	0.923	0.987	0.494	0.562
Min	0.312	0.483	0.119	0.246
Max	1.741	1.503	1.008	0.911
Patients (Acral. Growth)	82	104	----	----
Mean	1.373	1.324	----	----
SD	0.264	0.265	----	----
Median	1.359	1.323	----	----
Min	0.862	0.821	----	----
Max	2.034	1.788	----	----

*: Both RecGH and PitGH concentrations were greater than 0.09 ng/ml.

2) Difference of Ratios between Patients (Acromegaly/Pituitary Gigantism/Pituitary Adenoma)

and Healthy population: As can be seen in Tables 2, 3 and 4, the patients showed significantly higher ratios than healthy subjects; in some cases (18 sera in this study), the ratios were even higher than WADA criteria (> 1.68 for females). Some authors have shown that different patients may have different proportions of non-22 kDa hGH. For example, the authors [5] indicated the proportion of the smaller molecular isoform of hGH altered in patients with acromegaly. Other authors [6] reported that the percentage of non-22kDa GH isoforms was higher in both short children born small and those with Turner's syndrome than in normal children, suggesting a possible cause for growth failure with the abnormal increment in the circulation of non-22kDa GH isoforms. In the present study, however, it has been demonstrated, by direct 20kDa measurement, that the proportion of 20kDa does not vary according to age, puberty, or sex in normal children, and that it also does not correlate with the height sd score in normal children or in children with growth disorders. Some authors recognized [9] that **"**this molecular heterogeneity appears to have physiological significance, as different forms have been shown to possess different biological activities and immunodetectabilities".

3) Concentrations Measured by Kits A and B: As shown in Table 1, the variation of quality controls in Kit A, even with very low concentrations of recGH or pitGH, was very small. In our results, some correlation between Kits A and B was observed and recGH A was slightly higher than recGH B, but pitGH A was much smaller than pitGH B. This may be the reason why ratio A was more likely to be greater than ratio B. Obviously, the antibodies recognize the pitGH isomers to a different extent due to the heterogeneous nature of pitGH. It was suggested by the authors [8] that **"**A single reference preparation used for assay calibration should be universally adopted. It should be chemically defined." "Where isoform-specific assays are available (20 kDa GH, GH-V), they should be used (this currently applies to research only)." Interestingly, it is also noted that the ratios measured by Kit A was statistically about 1.7 to 1.8 times higher than that measured by Kit B for both females and males (refer to Table 4 and Table 6). In fact, WADA criteria for Kit A were about 1.3 to 1.4 times higher than that for Kit B. We ask whether further investigation of population based statistics about the ratio of values obtained with Kit A and Kit B is needed?

4) Concentrations Measured by Different Commercial Kits: The correlation between anti-doping test Kits and clinical test Kits was evaluated. The sum of concentrations of recGH and pitGH measured by Kit A in Group B correlated quite linearly with GH concentrations measured by the DPC GH test Kit as shown in Figure 2. It was clear that the correlation between different kits was much better in the lower concentration range than in the higher range.

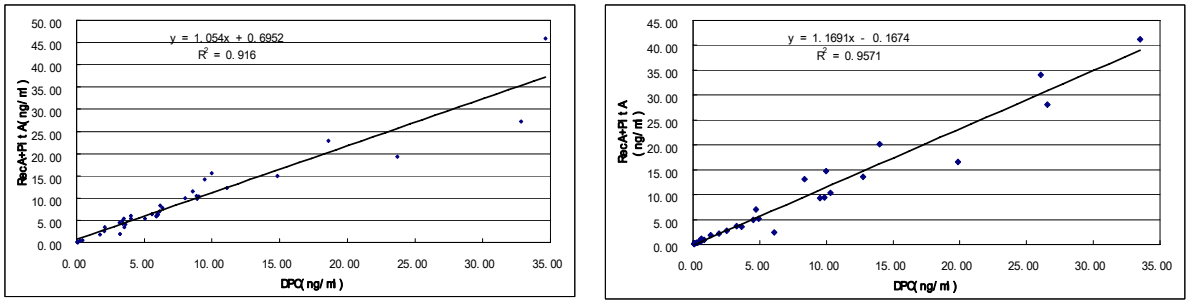


Figure 2 The Correlation of GH Concentrations Measured by Different Test Kits (left: female; right male)

In contrast, the sum of concentrations of recGH and pitGH measured by Kit A in Group A was not well correlated linearly with GH concentrations measured by DPC GH test Kit (not included in this paper) as being shown in Figure 2, probably due to the patients (Acromegaly/Pituitary Gigantism/Pituitary Adenoma) excreting high levels of GH potentially from different organs/cells.

5) Detection of Doping with rhGH: Though the detailed information for Group B was not provided, the two horizontal lines in Figure 1 indicated that around 50 % of the patients with administration of rhGH could be declared using WADA criteria.

Conclusions

1) Further population studies are needed to investigate the GH isoform ratios of females and males and to evaluate whether the ratios are significant different between genders.

2) More assays are required with the aim of investigating the difference between Kit A and

Kit B discussed herein to predict whether this difference has to be taken into account for redefining the thresholds for Kit A and Kit B, respectively.

3) Intensive study is still needed to gain sufficient information on the pathological conditions affecting the ratios of recGH to pitGH to determine whether medical tests, such as the glucose-tolerance test etc., is required to exclude any pathological effect on the ratios that are potentially reported as an analytical adverse finding.

Acknowledgements

The authors are in indebted to Prof. Dr. David Cowan and Dr. Christian Reichel for valuable discussions during the Beijing Games, to Dr. Olivier Rabin and Dr. Osquel Baroso for organizing the implementation of GH test and to Chinese National Science Foundation (contract number 20635010) and National Key Technologies Research and Development Program (code 2003DA904B05) for financial support.

Special thanks to Prof Dr. David Cowan for both his valuable scientific discussion and correction in English wording.

References:

1) Bidlingmaier M, Suhr J, Ernst A, Wu Z, Keller A, Strasburger CJ, Bergmann A. High-sensitivity chemiluminescence immunoassays for detection of growth hormone doping in sports. *Clin Chem.* 2009 Mar;55(3):445-53. (with Suppl.)

2) Irie M, Ueki M, Kishikawa Y, Nishii M, Kawahara T. 20K-GH and its use in detecting GH abuse. *Growth Hormone & IGF Research* (2009), doi:10.1016/j.ghir.2009.04.013

3) Engstrom BE, Karlsson FA, Wide L. Gender differences in diurnal growth hormone and epinephrine values in young adults during ambulation. *Clin Chem.* 1999;45:1235–1239.

4) Ho KY, Evans WS, Blizzard RM, et al. Effects of sex and age on the 24-hour profile of growth hormone secretion in man: importance of endogenous estradiol concentrations. *J Clin Endocrinol Metab.* 1987;64: 51–58.

5) Zhang Xiulan Deng Jieying Jin Zimeng Shi Yifan. Heterogeneity of serum human

growth hormone in acromegaly before and after treatment. Department of Endocinology , Capital Hospital, Chinese Academy of Medical Science. *Chinese Journal of Endocrinology and Metabolism* 1985 Vol 1(1): 17-20

6) Boguszewski CL, Jansson C., Boguszewski MCS., Rosberg S., Carlsson B., Albertsson-Wikland K., Carlsson LMS. Increased proportion of circulating non-22-kilodalton growth hormone isoforms in short children: a possible mechanism for growth failure. *J Clin Endocrinol Metab.* 1997;82:2944 –2949

7) Lewis UJ, Markoff E, Culler FL, Hayek A, Vanderlaan WP. Biologic properties of the 20K-dalton variant of human growth hormone: a review. *Endocrinol Jpn*. 1987; 34(Suppl 1):73– 85

8) Popii V. and Baumann G., Laboratory measurement of growth hormone, *Clin. Chim Acta* 2004, 350:1-16

9) Growth hormone Physiological Effects of Exogenous Administration in Performance-Enhancing Substances in Sport and Exercise, M.S.Bahrke and Ch.EYesalis (Edit), Human Kinetics, 2002 p. 65-78