Research article

Drug Testing and Analysis

Received: 15 June 2012 Revised: 4 September 2012 Accepted: 4 September 2012 Published online in Wiley Online Library: 16 October 2012

(www.drugtestinganalysis.com) DOI 10.1002/dta.1423

Biomarker detection of rhGH Doping: an excretion study

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The purpose of this research is to validate the biomarker-based approach for the detection of doping with recombinant human growth hormone (rhGH) in sport. The GH-2000 project proposed an indirect method for the detection of exogenously administered growth hormone (GH) based on the measurement of the GH-dependent markers: insulin-like growth factor-I (IGF-I) and Type III pro-collagen (P-III-P). These markers rise in a dose-dependent manner after GH application. In this study, the concentrations of IGF-I, IGF-BP3, and P-III-P in serum were determined to provide further incentives for the implementation of this detection assay in modern anti-doping programmes. This paper reports on an administration study of rhGH involving 25 Chinese male volunteers at a dose of 0.1 IU /kg/day for a continuous 14-day period.

We observed that the serum IGF-I concentration increased rapidly in the rhGH treatment group and showed significantly higher levels compared to baseline between days 4 and day 16 after administration. Although the response of P-III-P to rhGH administration was delayed compared to the IGF-I axis, the P-III-P concentration remained increased for a longer period (from day 4 to day 28). Statistical analysis was carried out to establish a discriminant formula with Statistical Product and Service Solutions (SPSS) concluding that the biomarker methodology is valid and universally applicable. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: anti-doping analysis; indirect method; IGF-I; P-III-P; discriminant formula

Introduction

Recombinant human growth hormone (rhGH) is considered as an effective hormone for the enhancement of an athlete's physical performance.[1–3] So far, two different methodologies (referred to as isoform and biomarker test) have been developed for the detection of rhGH.[4,5] Researchers continue to identify novel tests and additional indicators for the detection of rhGH; genomic and proteomic approaches are being explored to find indicative information of rhGH abuse,[6–8] in addition to alternative methods to detect growth hormone releasing peptides (GHRP) and its metabolism. The latter, GHRPs, are prohibited in sports as releasing factors of GH and masking agents.[9,10]

The determination of rhGH using the isoform method, approved by the World Anti-Doping Agency (WADA) in routine analysis,[11] has a short detection window of approximately 1 day after the last rhGH injection. In a previous paper on rhGH excretion by Jing *et al.*, the detection window was determined to be no longer than 21 h.[12] Only a few adverse analytical findings using this method have been recorded worldwide. Due to its short detection window, the isoform test is more suitable for out-of-competition testing.

The indirect method or 'markers approach' refers to measuring the changes of several serum proteins as biological markers. Some of these markers are more stable and have a longer half-life than GH itself, which could exhibit better stability in serum during a prolonged period. Studies devised by the GH-2000 and GH-2004 projects[13–15] together with authors from Kreischa proposed or modified the discriminant formula with the combination of IGF-I, P-III-P, and other markers to detect rhGH doping.[16–18] These studies, however, involved predominantly white European (Caucasian) athletes with limited Chinese subjects and thus have limitations on the relevance of rhGH administration in Chinese people.[5,19]

This paper presents and discusses the detection of rhGH using the indirect biomarker method in healthy Chinese males. The results indicate that the markers serve as an important complementary test to the WADA-approved isoform test, providing independent information, and a longer window of detection. The in-house validation of this method is part of a crucial strategy to improve the sensitivity and specificity of markers approach as a reliable GH detection test.

Experiments

Excretion study

This excretion study was double blind, randomized, and placebo controlled as reported in our previous paper.[12] Twenty-five male students (non-athletes, age 24.0 ± 1.35 years, body mass index 22.2 ± 2.26 kg/m^2) were randomly assigned to one of three treatment groups: the placebo group, the Chinese rhGH preparation (GenHealW, S19990019; code of Chinese State Food and Drug Administration approval (SFDA)); and the Swiss rhGH preparation (Saizen®, S20080036 (code of SFDA approval)). Administration was at 0.1 IU/kg body weight by one subcutaneous injection daily on the different areas of abdomen for 14 days, followed by a

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3-week wash-out period. Basal samples were collected before the first injection (0 h). The first administration took place at 8 am with all subsequent administrations at 9 pm. Then 6 blood samples were collected 2, 4, 8, 12, 23, and 34 h after the first injection. The remaining blood samples were collected either 10 h or 21 h after the injection. During the wash-out period, blood samples were collected for the first 4 days after the last injection and subsequently on day 21, day 28, and day 35. Blood samples were collected and stored following WADA guidelines.[11]

Analytical procedure

All blood samples were centrifuged immediately after the sample was taken. The separated serum of each sample was stored at -70°C until analysis.

Serum concentrations of IGF-1 and IGFBP3 were measured using the Immulite 1000 with commercially available kits from Siemens, Germany. Serum P-III-P was determined by radioimmunoassay (RIA) manufactured by CIS Biointernational (Gif-sur-Yvette Cedex, France) and by UniQ P-III-NP RIA (68570) from Orion Diagnostica (Espoo, Finland). All RIA analyses were undertaken in the ISO 15189:2007 and NCCLI EQA certified Institute for Clinical Diagnosis in Beijing. P-III-P was analyzed in house using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) analysis (TPI Inc., Lynnwood, Washington, USA).

The analysis was in duplicate manner with a CV% less than 25% for radio-immunoassays and 20% for the other immunoassays. All samples from each volunteer were analyzed in one run to avoid the inter-variation between batches.

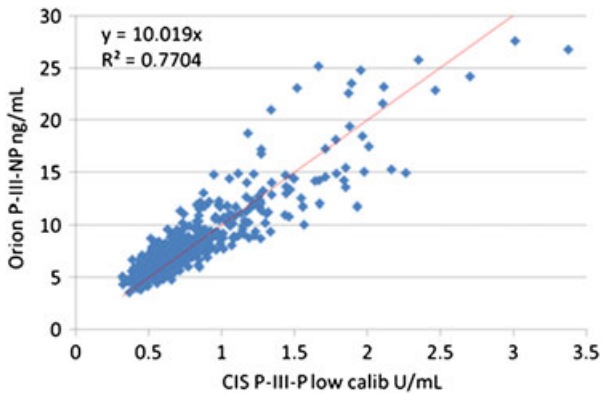

Figure 1. Cross-correlation of the assays for P-III-P (Orion and CIS).

Statistical analysis

Statistical analysis was performed using the SPSS v.13.0 (SPSS Inc., Chicago, Illinois, USA) under the consultation and supervision of Dr Zhao Shuxiang, a mathematician at the Beijing Sport University. All analyses were performed on the logarithmical transformed values of IGF-1 and P-III-P. Placebo and pretreatment basal data of the treatment group were used as negative class, while the data from the administration group between day10 and day 14 were considered as positive class.

Results and discussion

Cross-correlation of different assays for P-III-P

As there are no P-III-P International Standards or International Reference Materials for ethnic variations of P-III-P measurements, the comparison of the concentrations detected by different assays was performed. CIS Biointernational assay (France) reports P-III-P levels in units (U/ml), while the Orion assay (Finland) reports in ng/ml. Also the ELISA (TPI Inc., Lynnwood, Washington, USA) test reports P-III-P concentrations in ng/ml. The cross-correlation of different assays for P-III-P is presented in Figure 1. Data derived from CIS and Orion have linear correlations ($r = 0.794$), but no significant cross-correlation is observed between any of two RIA assays and the ELISA test (Figure 2).

The WADA code requires that for each analyte measured by immunoassay, different assays must be used for screening and confirmation; this means that the screening and confirmation assays must recognize different epitopes of the analyte. The strong positive correlation between values measured by CIS and Orion may indicate that the two different assays could be adopted for screening and confirmation purposes in future detection methods. The correlation ($y = 10.019\ x$, $R^2 = 0.7704$, $n = 499$) between CIS and Orion assays in our study concurs with data reported in the literature ($y = 9.2057x$, $R^2 = 0.7676$, $n = 124$).[20] Furthermore it confirms that the stability of these two P-III-P tests is appropriate for the biomarker approach in the anti-doping field.

Effects of rhGH on serum IGFI,P-III-P and IGF-BP3 levels

The basal concentrations of IGF-I, P-III-P and IGF-BP3, measured by different assays have no significant differences ($P > 0.05$) in the placebo and preparation groups (Table 1).

As shown in Figure 3, the IGF-I levels increased rapidly after administration. Data from day 4 (around 100 h) to day16 (around

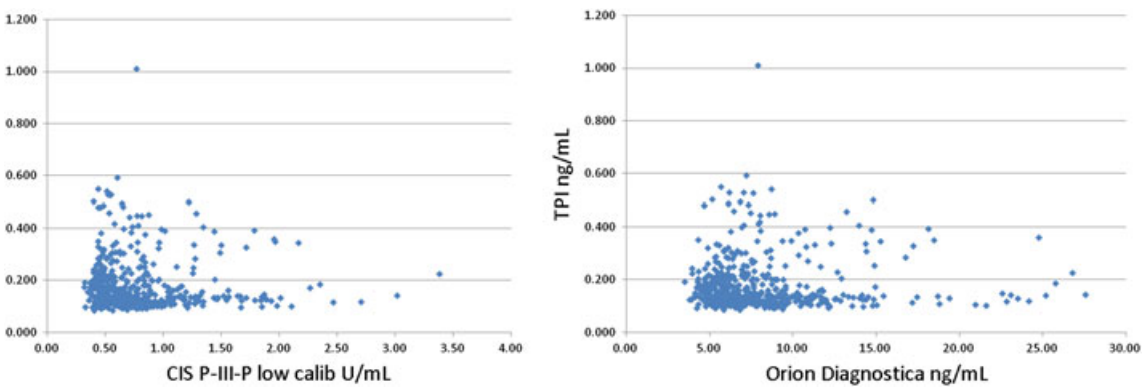

Figure 2. Cross-correlation of the assays for P-III-P (RIAs and Elisa).

400 h) after the first injection were significantly higher than the baseline ($P < 0.01$) and placebo groups ($P < 0.01$). The maximum concentration value on day 10 (around 230 h) diminished to the baseline on day 18 (4 days after discontinuation of injection). It is interesting to report the following: though rhGH was given to the volunteers with a daily dose of 0.1 IU/kg after achieving the peak concentration ~700 ng/ml, ~100 h after the first injection, the IGF-1 concentration continually decreased. Two days after the last administration, IGF-1 was observed at baseline levels. The administration groups (with two different preparations) demonstrated similar decay rates.

Table 1. Basal values of the volunteers

Measurements	Chinese Prep. Group(n = 13)	Swizerland Prep. Group (n = 6,)	Plecebo Group(n = 6)
IGF1(ng/ml, Germany)	243.23 ± 52.00	236.17 ± 69.42	263.60 ± 29.54
IGFBP3(ng/ml, Germany)	5.03 ± 0.62	5.22 ± 0.62	5.46 ± 0.52
P-III-P(U/ml, France)	0.48 ± 0.07	0.45 ± 0.04	0.48 ± 0.05
P-III-P(ng/ml, Finland)	6.00 ± 1.19	5.37 ± 0.39	6.07 ± 0.97

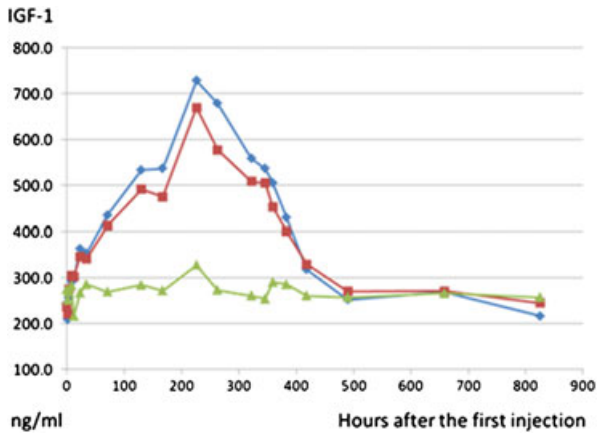

Figure 3. Time course of mean IGF-1 after rhGH injection (Green: placebo, blue: administration group having received the Chinese rhGH preparation, brown: administration group having received the Swiss rhGH preparation).

Figure 4 shows the response of P-III-P to rhGH injection measured with two different assays (Orion and CIS). P-III-P concentrations show similar trends measured by these different RIA assays. From day 4 to day 28 after the first injection, the P-III-P levels in the group administered with the Chinese rhGH preparation significantly increased compared to the placebo group ($P < 0.05$). The same increase is observed earlier in the group administered with Swiss rhGH preparation from day 12 to day 21. P-III-P concentrations have little fluctuation in respect to the baseline on day 35. Data from the ELISA assay has no significant change in respect to the baseline and placebo groups.

The IGFBP3 response is lower than the increments of IGF-I. As showed in Figure 5, from day 6 to day 15, the IGFBP3 level in the administration groups are slightly increased. However, the IGFBP3 levels in the placebo group do not show significant difference from the administration groups. As daily variations in IGFBP3 levels are observed, it was determined not to include the concentration of IGFBP3 in the statistical analysis.

Discriminant function formula

Based on our excretion study data, discriminant formulae are proposed by logistic regression analysis involving IGF-I and P-III-P that are comparable to those derived from the GH2000 project. As the ages of all volunteers in this excretion study show no significant difference, age-related factors are excluded from the discriminant formulae. As all volunteers were male, gender variables were also excluded.

Because the P-III-P concentrations are determined by Orion and CIS,the two discriminant formula are as follows:

$$\text{Orion Score} = 3.367 \times \ln(\text{IGF1}) + 3.446 \times \ln(\text{P-III-P}) - 24.715 \quad (1)$$

$$\text{CIS Score} = 3.812 \times \ln(\text{IGF1}) + 2.800 \times \ln(\text{P-III-P}) - 19.384 \quad (2)$$

Orion and CIS scores were calculated using all IGF1 and P-III-P concentrations (n = 25) through the above respective formulae. Figure 6 illustrated that two administered groups (n = 19) show significant difference in either Orion or CIS scores, compared to the placebo group (n = 6).

Evaluation of the discriminant functions

The rhGH scores in the administration groups increased rapidly from day 2 after the first injection, and start to decrease on day 18. From day 2 to day 28 (2 weeks after discontinuation), the

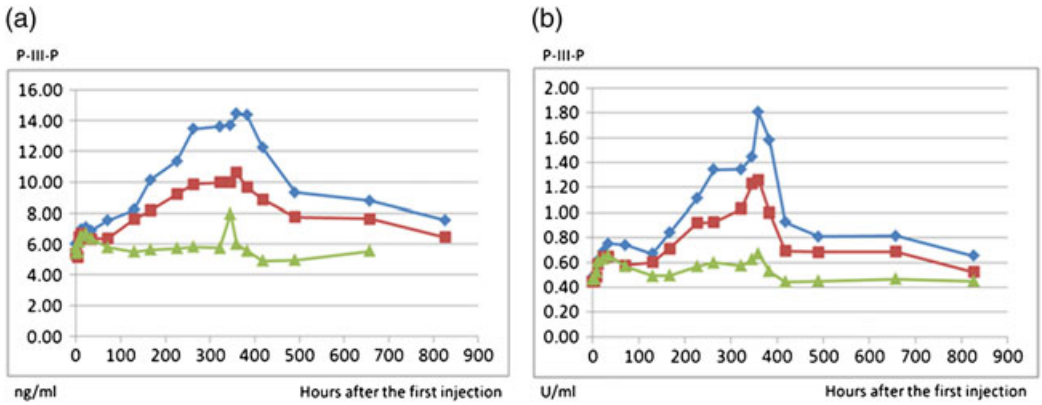

Figure 4. Time course of mean P-III-P after rhGH injection measured with Orion and CIS (Green: placebo, administration group having received the Chinese rhGH preparation, brown: administration group having received the Swiss rhGH preparation. (A): Orion, (B): CIS).

rhGH scores of the application groups show significant difference compared to the placebo group.

The scores, calculated against the samples, including samples of placebo group and basal samples of administration groups, show a normal distribution. Their scores therefore have a mean of 0 and standard deviation of 1 by definition. A score of 3.7 is equivalent to the 99.99% reference limit. It has been suggested that a possible cut-off point for detection of rhGH usage for this study should be set at 3.7, enabling a false positive rate of no more than 1:10000[5]. In a recent paper, different opinions regarding the limit of 3.7 are reported.[21] As debates surrounding this limit are yet to be concluded this paper is only concerned with the agreed limit of 3.7. Figure 7 shows the distribution of these scores in agreement with the published literature.[5]

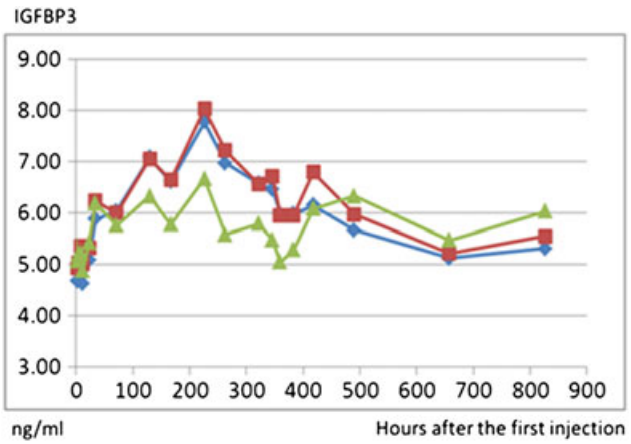

Figure 5. Time course of mean IGFBP3 after rhGH injection (Green: placebo, blue: administration group having received the Chinese rhGH preparation, brown: administration group having received the Swiss rhGH preparation).

The sensitivity of formulae for finding 'positives' on a distinct day is defined as the number of observations placed over the 3.7 cut-off value out of the total number of observations for the particular day from volunteers in two treatment groups. A sensitivity of 63.2% (12/19) is found from day 10 to day 16 of the rhGH application, the maximum of 78.9% (15/19) is observed on day 10. There are still 47.4% (9/19) of those treated with rhGH that are identified as positive on day 17 (3 days after the last injection) with the discriminant formula reported in this paper; no discriminant formula allowed for the determination of an adverse analytical finding after day 21.

With a cut-off score of 3.7, rhGH doped samples can be detected from day 6 (after the first injection) to day 18 (3–4 days after discontinuation) with 14 days injections at a rate of one per day.

As reported previously, the detection of rhGH using the isoform method provides a detection widow of no longer than 24 h, in either single or multi-dose administrations. By comparison, the markers approach has the potential to improve upon the window of detection thus improving on the capabilities of the isoform method.

Validation of the markers approach with independent data sets

The complexity of rhGH detection became more apparent when the GH-2000 and Kreischa discriminant formulae were applied to alternative data sets.[5] The results revealed that when GH-2000 data were applied to both GH-2000 and Kreischa formulae, the Kreischa function exhibited much lower sensitivity. Similarly, when the Kreischa-derived data were applied to the GH-2000 and Kreischa formulae, the Kreischa function yielded much better results than the GH-2000 formula. In both research groups, discriminant formulae are derived from own data sets resulting in a particular 'data-formula-score' system performance, meaning that the Kreischa formula is better suited for Kreischa

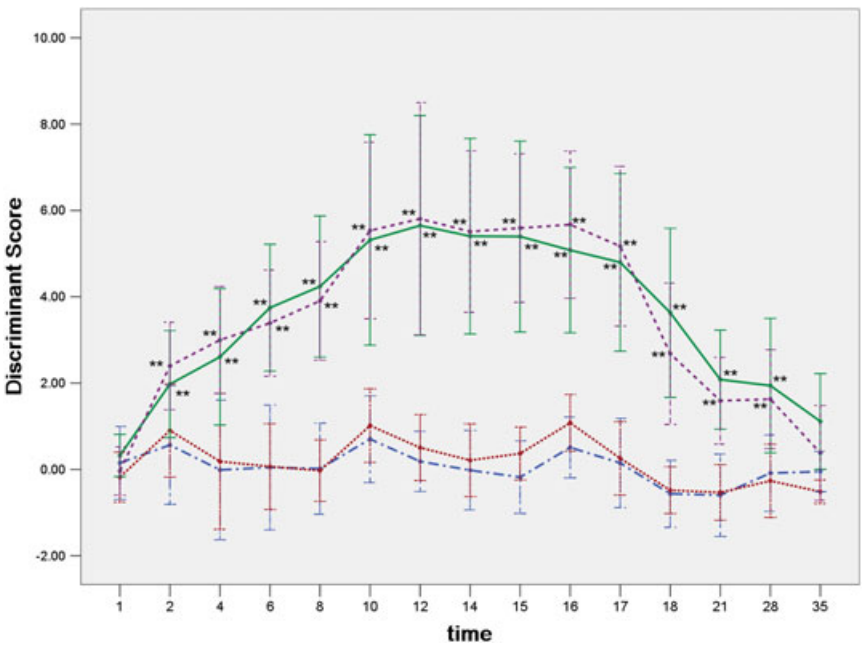

Figure 6. Time course of the scores expressed in mean ± SD (Solid line: Orion score of administration study group, dash spot line: Orion score of placebo group; double dash spot line: CIS score of administration study group, dash line: CIS score of placebo group, time in day).

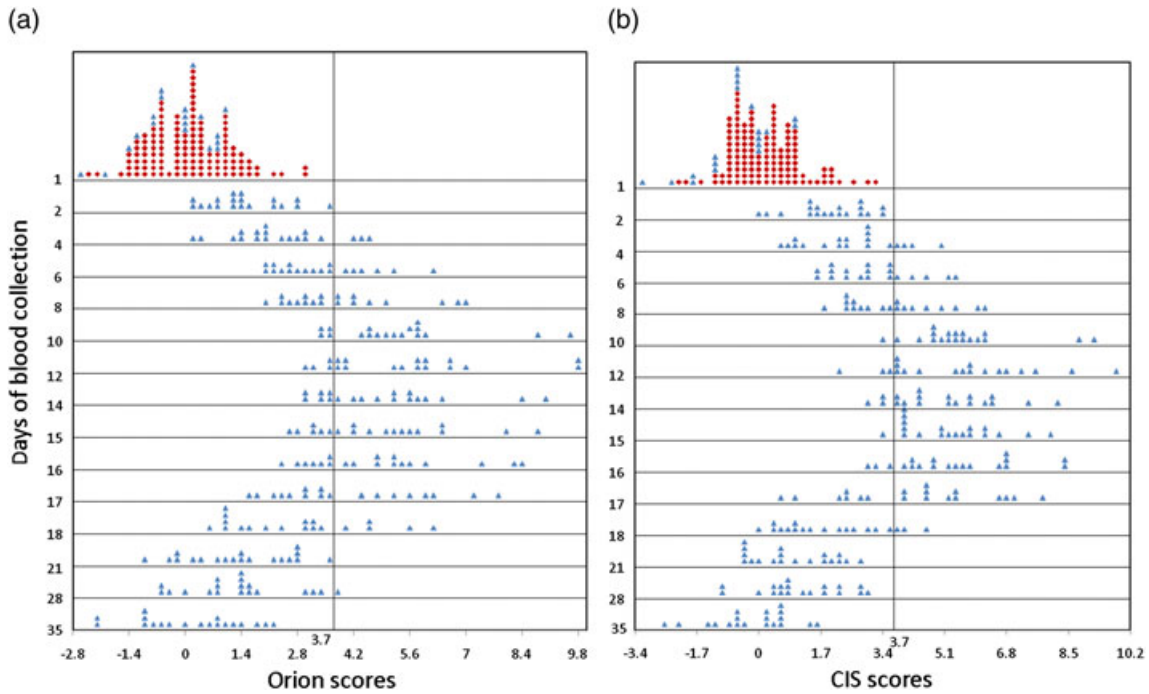

Figure 7. Distribution of the scores ((A): Orion score, (B): CIS score).

administration study data. The balance between the sensitivity and specificity of the methodology is the most important factor to be considered and the in-house validation is the key point for the implementation of the biomarker approach. The IGF-1 levels in our study were measured with the Immulite 1000, while those in the GH-2000 project used a radioimmunoassay (RIA) assays, both measuring ng/ml.

In agreement with the assays in the GH-2000 project, both Kreischa and our administration study were analyzed for P-III-P levels. The basal data have no significant difference ($P > 0.05$), the GH-2000 formula without age correction was applied to the data (IGF-1 and P-III-P) from our excretion study. The results in Figure 8 show less sensitivity for catching the positive case than the data expressed in Figure 7. The formula for the scores in Figure 8 was reported by GH-2000 group in the literature.[5]

The functional abilities of the formulae diminish when applied to different data sets other than their own. Possible reasons may be a different dosing applied to study groups, different ethnicities adopted in the GH-2000 project as well as the Kreischa and our study, etc. Accordingly, further in-house validation and conversion of the formulae between different studies may be needed for implementing the markers approach.

Other factors effecting discriminant functions

Although markers such as IGF-I and P-III-P are more stable in serum than GH itself, they can vary widely between individuals, depending on age, sex, body weight, physical activity, diet, and androgen or oestrogen use. These variables introduce difficulties in defining appropriate cut-off levels beyond which rhGH doping can be proven. During our study, discriminant formulae are derived from non-athletes. It is not yet known how well these formulae will perform in real life where the patterns and doses of GH abused by athletes are unclear.

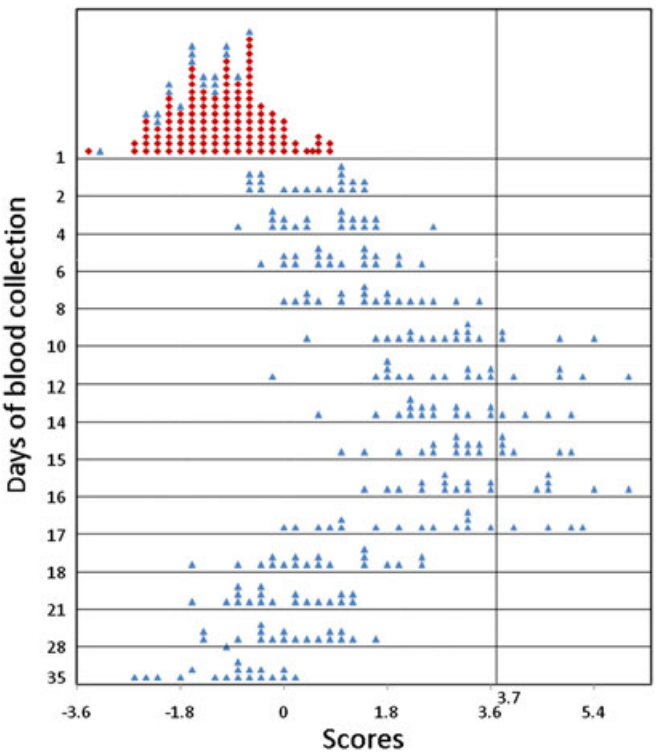

Figure 8. Distribution of the GH-2000 scores with the data obtained in this study.

In our study only males of very similar age were included, so the difference between genders and ages were not investigated. These effects should be studied in future validation for routine work.

Conclusion

In summary, this excretion study has confirmed that serum IGF-I and P-III-P levels increase in response to rhGH administration in healthy Chinese males. Discriminant formulae are proposed to detect the rhGH use, which could be a complementary approach for isoforms method in future tests.

The biomarkers approach for the detection of doping with rhGH showed a longer time window than that of the isoform approach. Our excretion study confirmed that principally the GH-2000 methodology is universal.

Based on the data of our excretion study, the capability of the in-house formula was comparable to those of the GH-2000. Further investigation is required for the Decision Limit relevant to different data sets using same kits for better data harmonization is recommended.

Acknowledgements

We give special thanks to Prof. Dr Jin Zimeng at the department of endocrinology of Peking Union Hospital for his valuable consultation, to Shanghai United Cell Biotechnology CO. Ltd for the donation of rhGH preparations and to Dr Zhao, Shuxiang at the Beijing Sports University for the consultation and suggestion on statistical analysis.

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