Review

Drug Testing and Analysis

Received: 7 May 2009 Revised: 5 August 2009 Accepted: 19 August 2009 Published online in Wiley Interscience: 26 October 2009

(www.drugtestinganalysis.com) DOI 10.1002/dta.59

Detecting growth hormone abuse in athletes

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There is widespread anecdotal evidence that growth hormone (GH) is used by athletes for its anabolic and lipolytic properties. Although GH is on the World Anti-doping Agency (WADA) list of banned substances, the detection of abuse with GH is challenging. Two approaches have been developed to detect GH abuse. The first is based on the measurement of pituitary GH isoforms and the second is based on the measurement of markers of GH action.

Pituitary GH contains multiple isoforms whereas recombinant human GH comprises solely the 22-kDa isoform. Immunoassays that recognize the different isoforms have been developed and form the basis of the test introduced by WADA at the Athens Olympic Games. To date, no athlete has tested positive.

The GH-2000 project proposed a test based on the measurement of insulin-like growth factor-I (IGF-I) and type III pro-collagen (P-III-P) as these markers increase in a dose-dependent manner in response to GH and their basal concentration varies much less than GH. When combined with discriminant function analysis, these markers were able to differentiate between those taking GH and placebo in double-blind placebo controlled trials. Subsequent studies have shown that the test is applicable across different ethnicities and is unaffected by injury.

Keywords: abuse; biomarker; GH; IGF-I; isoforms; P-III-P; sport

Introduction

Growth hormone (GH) is a naturally occurring peptide hormone produced by the pituitary gland. It is believed that human (h)GH has been misused since the 1980s by sportsmen and women who have sought to exploit its anabolic and lipolytic actions to achieve a performance benefit.[1] Its use was prohibited by the International Olympic Committee (IOC) in 1989 and it appears on the current World Anti-Doping Agency (WADA) list of prohibited substances.[2] As early as 1992, the International Olympic Committee (IOC) realized that the issue of GH doping required an effective test. This review will discuss the challenges involved in the detection of GH misuse and the work undertaken to develop a test to detect its abuse.

Challenges in the Detection of GH Abuse

The detection of doping with exogenous GH poses a formidable challenge for several reasons. It is difficult to differentiate between recombinant (r)hGH and endogenously produced pituitary GH as rhGH has an identical amino acid sequence to the native 22 kilo Dalton (kDa) hormone. Cadaveric GH contains the full range of GH isoforms and so is indistinguishable from endogenous GH. In view of this, demonstration of exogenous administration must rely on detecting levels in excess of those found in normal physiology. This in turn creates a further challenge because GH has a short half life (<20 minutes) and is secreted in a pulsatile manner leading to wide variations in circulating GH concentration during the course of the day. There are many physiological factors that regulate GH secretion but in the context of anti-doping it is important to recognize that both exercise and stress are powerful stimuli for GH secretion.[3,4] The normal response to exercise is a brisk and marked increase in GH secretion. Consequently the finding of a high GH concentration in the post-competition setting may merely reflect endogenous secretion.

This is well recognized in clinical endocrinology where a diagnosis of acromegaly, a condition usually caused by excessive secretion of GH from a pituitary adenoma, cannot be made by a single GH measurement. Acromegaly is diagnosed by assessing the response of GH to the administration of an oral glucose load over a two-hour period. This test may be supplemented with repeated measurements of GH throughout the day. These are effective ways of diagnosing acromegaly but they are impractical for anti-doping purposes, where repeat sampling is not possible.

The mass spectroscopy methods for detecting the abuse of androgenic anabolic steroids and related substances in urine are highly sophisticated but such methods to detect GH are in their infancy. A major problem in the analysis of urinary GH is that the urinary clearance of GH is not a constant function of plasma GH.[5] The rates of glomerular filtration and tubular reabsorption determine urinary clearance and GH reabsorption from the glomerular filtrate is sensitive to ambient protein concentration in the filtrate and this may vary widely.[6,7] Exercise increases urinary protein excretion and this inhibits GH reabsorption leading to a significant increase in urinary GH concentration.[8] Consequently, immunoassays and blood sampling are currently preferred for the detection of these substances.

In addition to these biological considerations, there are a number of practical aspects to any doping test. Ideally it should be cheap, have a high-volume testing capacity and ease of technical operation with a high sensitivity and specificity in order to avoid making false accusations.[9] Finally it must also be acceptable to the sporting community and easy to administer track side.

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Development of a Test to Detect Growth Hormone Abuse

Two different yet complementary approaches have been investigated to detect GH abuse; the first, pioneered by Christian Strasburger and Martin Bidlingmaier in Germany, is based on the detection of different pituitary GH isoforms[10,11] whereas the second uses the measurement of GH-dependent markers.[1,12]

The isoform or differential immunoassay method

Endogenous pituitary GH exists as multiple isoforms; 70% of circulating GH is in the form of a 22 kDa polypeptide whereas 5–10% occurs as a 20 kDa isoform as a result of mRNA splicing. There are also dimers and oligomers as well as acidic, desaminated, acylated and fragmented forms of GH.[13]

By contrast rhGH comprises solely the 22 kDa isoform. When rhGH is administered, endogenous pituitary secretion is suppressed through negative feedback and so the ratio between 22 kDa and non-22 kDa isoforms changes, with 22 kDa hGH becoming predominant. The isoform method relies on the measurement of GH isoforms by two immunoassays that employ monoclonal antibodies with preferential binding to either 22-kDa GH or pituitary-derived hGH.[10,11,14] By examining the ratio of the results of the two assays, it is possible to differentiate between those receiving exogenous GH from those with endogenous secretion (Figure 1).[15]

Age, sex, sporting discipline and pathological states do not appear to affect the relative proportions of GH isoforms,[16,17] but exercise causes a transient relative increase in the 22 kDa isoform, thereby lowering the sensitivity of the test if samples are taken immediately after competition.[18,19]

The change in isoform ratio was first shown in subjects with GH deficiency receiving rhGH replacement whose ratio between 22 kDa and pituitary GH was greater than one while the samples from healthy control adults was less than one.[15] The effect on GH isoforms has subsequently been studied in an open label crossover study involving 10 healthy trained men and 10 women who received a single bolus injection of rhGH (0.033 mg/kg sc, 0.033 mg/kg im and 0.083 mg/kg sc) on three separate occasions.[20] Peak GH concentration and area under the curve were higher in men and following the intramuscular injection. In response to rhGH administration, 20 kDa GH decreased and remained suppressed for 14–18 hours in women receiving low-dose rhGH and 30 hours in the high-dose group. In men, 20-kDa GH was undetectable at baseline and throughout the study.

The effect of prolonged GH administration on the isoform ratio has been studied in an eight-week GH double-blind administration study undertaken in Australia but the results of this study have not yet been published in full.[21]

The WADA International Standard for Laboratories requires confirmation of any adverse analytical finding. For immunoassays, this means using a second set of immunoassays using antibodies that recognize different epitopes.[22] In order to comply with this, prior to the Athens Olympic Games, two pairs of 22 kDa and pituitary GH assays were developed in house. The initial versions were immunoradiometric assay (IRMA), which incorporated research-grade capture antibodies immobilized on the solid-phase surface of a micro-titre plate, a biotinylated detection antibody and a streptavidin–europium conjugate, which generated a signal that could be read using a fluorometer.[23]

The assays were validated in the Munich laboratory before being tested in WADA-accredited anti-doping laboratories in Sydney, London and Athens. Two External Quality Assessment

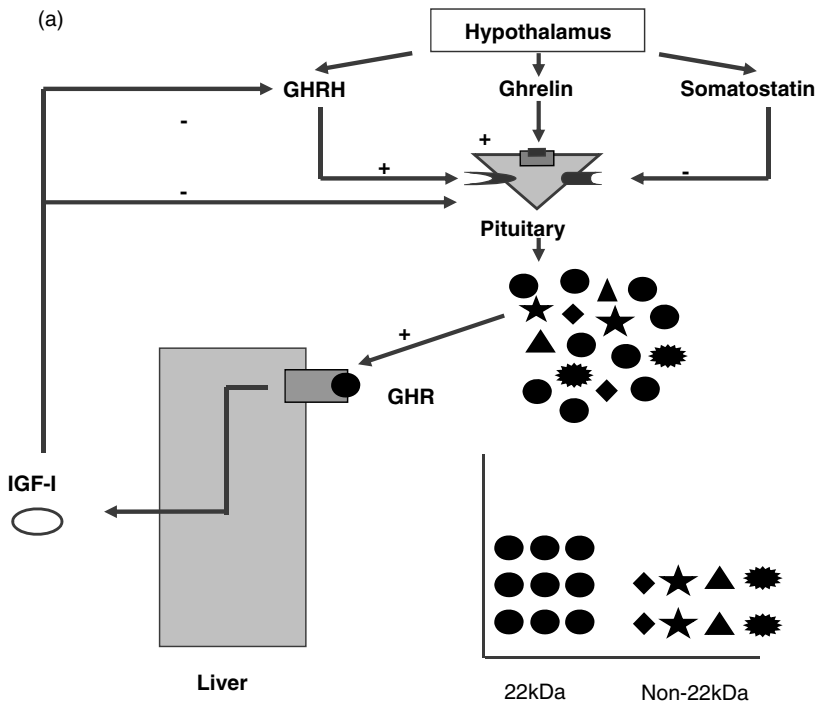

Figure 1a. The principle of the isoform method. Under normal circumstances, GH is secreted from the pituitary under the control of the hypothalamic hormones, growth hormone releasing hormone (GHRH), somatostatin and ghrelin, which is also produced by the stomach. Growth hormone appears as different isoforms, although 22-kDa represents the majority (represented in the graph and figure as a circle). IGF-I is secreted in the liver and acts in a classical negative endocrine feedback mechanism to reduce GH secretion.

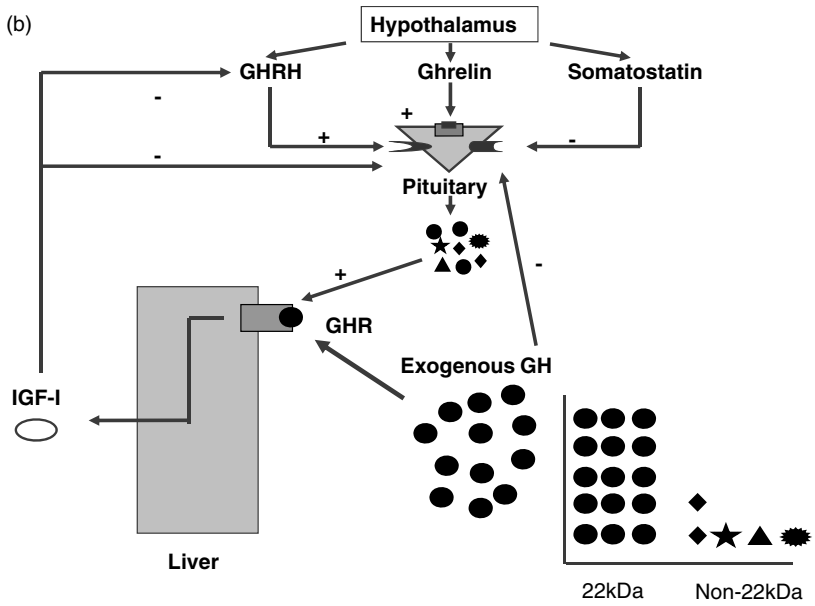

Figure 1b. When rhGH is administered, pituitary GH secretion is suppressed by negative feedback. Consequently the proportion of total GH which is 22 kDa increases.

Scheme (EQAS) rounds involving 27 samples from healthy young volunteers after injection of rhGH or placebo showed good consistency in the reporting of both negative and suspicious samples. No false positives were reported. The results were reviewed at a WADA sponsored GH Working Group in Dallas in April 2004 and the assays were approved for use at the Olympic Games in Athens 2004. The assays were subsequently used at the winter Olympic Games in Turin and the Commonwealth Games in Melbourne in 2006.[23]

It was recognized early in the assay development that, in order to maintain stability and reliability, the kits would have to be produced on an industrial scale under controlled manufacturing conditions. Initially Diagnostic Systems Laboratories (DSL) was approached to develop the assays but the collaboration was ended without fruition. Subsequently a contract was signed between WADA and SphingoTec GmbH, Berlin in December 2006 to adapt the assays to a new technical platform (tube-based chemiluminescence technique) that would be suitable for the production of commercial kits.[14]

This new platform is also an IRMA, but the capture antibodies are pre-coated on the surface of assay tubes and the detection antibody is directly labelled with acridinium esther, a chemical that gives a luminescent signal when excited at a specific energy in the reading instrument (luminometer). This development has significantly improved the detection sensitivity and kit stability as well as lowering the intra- and inter-assay CVs.

The progress of assay development, production and validation was reviewed at three meetings by WADA and United States Anti-Doping Agency (USADA) in Chicago (June 2007), Lausanne (November 2007) and London (April 2008, supported by UK Sport) prior to its use at the Beijing Olympic Games.[23] As yet the majority of the data incorporated in these reviews, including the population studies on which the cutoffs were derived, have not been published.

Despite nearly 1000 samples being measured, no positives have been detected in the post-competition setting. There are several reasons why this may have occurred, the main one being the relatively short window of detection of this test.[20] Recombinant hGH, even when injected subcutaneously, is cleared rapidly and GH is frequently undetectable in a blood sample taken the day after an injection.[24] Spontaneous GH secretion returns 48 hours after the last dose of rhGH treatment.[25] Consequently any athlete who ceases GH several days prior to the competition will not be detected. Thus the isoform method is unlikely to catch a GH abuser in the classical 'post-competition' dope-testing scenario and the optimal use of this method is likely to be in unannounced 'out-of-competition' testing. A further disadvantage of this method is that the use of cadaveric GH or GH secretagogues does not alter the isoform profile and so this test will not detect use with these compounds.

A slightly different approach has been taken adopted by an Australian-Japanese Consortium led by Professor Ken Ho in Sydney. This group has developed assays that specifically measure either 22 kDa or 20 kDa GH.[26] A pilot study showed that following the daily administration of 0.1 IU (0.033 g)/kg per day of 22-kDa-GH for 17 days, there was a rise in serum 22-kDa-GH concentration, which reached peak value 3 hours after the injection and returned to baseline by the following day. By contrast serum 20 kDa decreased before returning to the initial level at 24 hours. The ratio of 22-kDa GH to 20-kDa GH increased markedly following the GH administration but had returned to baseline within 24 hours in each of three subjects studied.[16]

The GH-dependent marker method

GH exerts its anabolic and metabolic actions in part through a change in the production of other proteins, some of which appear in the blood. Consequently the measurement of the concentration of one or more of these proteins may be a means of detecting exogenous GH (Figure 2). An ideal marker or combination of markers would have well-defined reference ranges, would change in response to GH administration and would remain altered after GH has been discontinued to provide a long 'window of detection' after administration.[17] The test should be validated across populations and should be largely unaffected by other

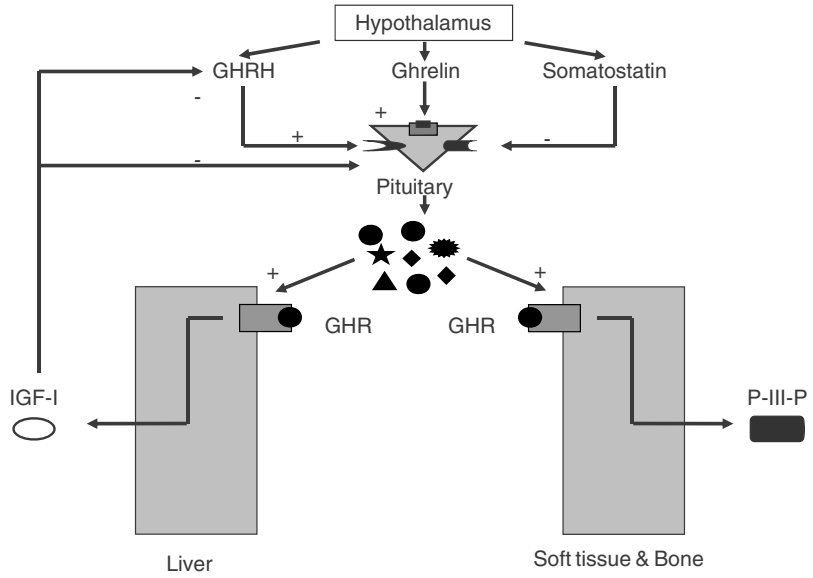

Figure 2. The principle of the marker method. Growth hormone acts on the liver to increase circulating IGF-I and on soft tissue to increase P-III-P.

Table 1. Characteristics of the GH-dependent markers chosen for in-depth study by the GH-2000 project

Marker	Abbreviation	Source	Physiological role	Disappearance half-time after GH (h)	Effect of Age	Diurnal variation	Effect of acute exercise	Important effect of ethnicity	Important effect of gender
Insulin-like growth factor -I	IGF-I	Liver	Mitogenic protein	89.5	↓	No	+20%	No	No
Insulin-like growth factor binding protein - 2	IGFBP-2	Liver	Regulates IGF-I bioavailability and action		↓	No	No change	No	No
Insulin-like growth factor binding protein -3	IGFBP-3	Liver	Regulates IGF-I bioavailability and action	176	↓	No	+18%	Yes	Yes
Acid Labile Subunit	ALS	Liver	Regulates IGF-I bioavailability and action	119	↓	No	+21%	Yes	Yes
Type III procollagen	P-III-P	Soft Tissue	Marker of soft tissue formation	693	↓	No	+5%	No	No
Osteocalcin	OC	Bone	Marker of bone formation	770	↓	No	No change	No	No
C-terminal propeptide of type I procollagen	PICP	Bone	Marker of bone formation	433	↓	No	+14%	No	Yes
Type I collagen telopeptide	ICTP	Bone	Marker of bone resorption	248	↓	No	+10%	No	No

regulators of GH secretion such as exercise, stress or injury. Ideally there should be no circadian or seasonal variation, no demonstrable difference between genders or menstrual cycle and the markers should exhibit low inter-individual and intra-individual variation.

Following his involvement with the IOC Medical Commission, Professor Peter Sönksen established the GH-2000 project, a large multi-centre project, funded by the European Union (under their BIOMED 2 initiative), the International Olympic Committee and the rhGH manufacturers Novo Nordisk and Pharmacia. The aim was to develop a test in time for the Sydney Olympic Games.

When the project was conceived, 25 potential markers of GH action were considered but with time these were whittled down to eight markers which were studied in greater depth (Table 1).[27,28] Finally two markers, insulin-like growth factor-I (IGF-I) and type 3 pro-collagen (P-III-P), were chosen for the GH-2000 test because they provided the best discrimination between individuals receiving GH or placebo during a randomized controlled GH administration trial, described below. Insulin-like growth factor-I is an ideal candidate marker as it has little diurnal or day-to-day variation,[29] rises post GH administration in a dose-dependent uniform fashion,[30] has a low basal scatter and only changes minimally with exercise.[28] Similarly P-III-P, which is a marker of type-3 collagen formation (mainly in soft tissues), exhibits little day-to-day, diurnal[31] or gender variation in basal concentrations. It rises, albeit more slowly than IGF-I, in a dose-dependent fashion following GH administration.[32] Although either of these markers could be used alone to detect

those taking GH, by combining markers in conjunction with the use of gender specific equations, 'discriminant functions', the sensitivity and specificity of the ability to detect GH abuse, can be improved compared with single-marker analysis.[12] A further advantage of this combination of markers is that IGF-I and P-III-P are produced in different tissues, thereby reducing the chance of a false positive (Figure 2).

The results of the GH-2000 project were reviewed critically by a group of experts at an IOC workshop in Rome in March 1999. The conclusion of the workshop was strong support for the methodology but several issues required further research before the test could be fully implemented at an Olympic Games. The major issue related to potential ethnic effects of GH as the vast majority of volunteers in the GH-2000 study were white Europeans. Secondly, as injury leads to alterations in collagen formation,[33,34] it was felt that injury may invalidate the test. The final consideration related to the feasibility of rolling the test out to anti-doping laboratories as the assays used in the GH-2000 study were manufactured commercially and therefore potentially subject to arbitrary changes by the manufacturers. The assays also employed radioisotopes for which many doping laboratories do not have a licence. Consequently, it was felt by the workshop that the IOC and subsequently WADA should develop their own immunoassays to address this.

The GH-2004 project based in Southampton was set up in December 2002 with the aim of addressing the questions arising from ethnicity and injury.

Reference Range for IGF-I and P-III-P in Professional Athletes

As stated previously, a demonstration of exogenous administration of GH relies on the detection of levels that exceed those that are found in normal physiology. The word 'normal' begs the question, 'what is ''normal'' in this context?' It is a reasonable hypothesis that athletes achieve their sporting successes by having a physiology that differs from people who are not professional athletes. Therefore it is important that a reference range for IGF-I and P-III-P is determined in elite athletes. The GH-2000 and GH-2004 studies have obtained blood samples within two hours of competition from over 1000 professional athletes from a number of sporting disciplines and differing ethnicities.[35,36] As in the general population, there is a marked decline in markers with age but ethnicity, sporting discipline, gender and body shape explained very little of the variability of the markers within the athletic population. The Australian-Japanese consortium has obtained blood samples from 1103 elite athletes out of competition and found similar findings in this setting. Less than 10% of the total variance of the markers was explained by gender, sporting discipline, ethnicity and body mass index, but age contributed to between 20–35% of the variance.[37]

Response of IGF-I and P-III-P

The response of IGF-I and P-III-P to GH has been determined in a series of placebo-controlled GH administration studies. The early work of the GH-2000 project was to examine the response of 25 potential markers of GH action to identify which markers were most suitable for a more in-depth analysis.[27,28] Recombinant hGH (0.15 IU/kg per day) was administered to recreational male athletes for one week with blood sampling during and after the GH administration. Eight markers from either the IGF-IGF binding protein (IGFBP) axis or markers of bone and soft tissue turnover were found to respond to GH administration and were studied in greater depth in a 28-day randomized double-blind placebo controlled GH administration study.[30,32] One or two doses of rhGH (0.1 or 0.2 IU/kg per day) or placebo were self-administered by 102 recreational athletes and the marker concentrations were measured before, during and up to 56 days after the discontinuation of the GH. Serum IGF-I increased in men receiving GH with no differences between the doses (Figure 3). There was a significant difference from placebo, which persisted to day 42, or two weeks after discontinuation of GH. By contrast, in women, the absolute IGF-I response was lower and there was a clear dose-response relationship. IGFBP-3 and acid labile subunit (ALS) concentrations increased in men whereas in women there was only a modest rise in ALS with no change in IGFBP-3. The IGFBP-2 levels decreased in response to GH administration in both men and women.[30] Type 3 pro-collagen increased in a dose-dependent manner and remained significantly higher in the GH treated groups up to six weeks after the cessation of GH treatment. Osteocalcin, C-terminal propeptide of type I procollagen and type I collagen telopeptide all increased in response to GH. Again, men were more responsive to rhGH than women.[32] Although several markers responded to GH, IGF-I and P-III-P were identified as those providing the best discrimination between individuals taking GH and individuals taking placebo.

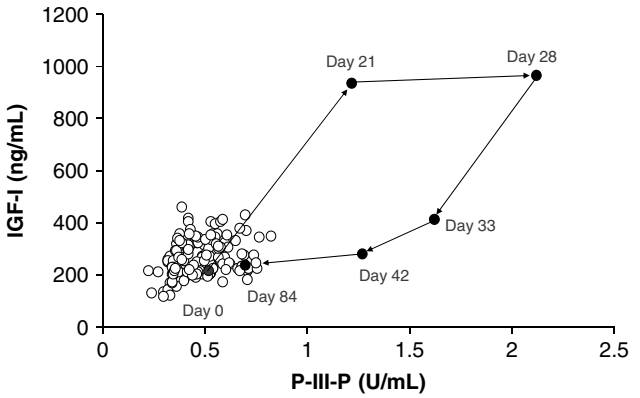

Figure 3. Change in IGF-I and P-III-P in one male volunteer following the administration of 0.2 IU/Kg/day for 28 days (solid circles). The values of the normal control men are shown in open circles. Note how the rise in IGF-I is quicker than P-III-P but P-III-P remains elevated for longer after the discontinuation of rhGH.

Since the publication of the GH-2000 studies, three other GH administration studies have been undertaken that provide further validity of the response of IGF-I and P-III-P.[21,38,39] The first study was performed at the Institut für Dopinganalytik und Sportbiochemie in Kreischa, Germany.[38] Amateur athletes received GH (0.06 IU hGH/Kg per day) for 14 days at a dose that was approximately half that used in the low-dose GH group in the GH-2000 administration study. Despite the lower dose, IGF-I concentration increased rapidly with rhGH and was significantly higher than baseline within three days of the start of treatment. The response of P-III-P also increased but, as was found by the GH-2000 group, it was delayed compared with IGF-I; by contrast, however, P-III-P concentration remained elevated for a longer period after discontinuation of GH.

The Australian group undertook an eight-week double-blind GH administration study in order to assess whether the GH-dependent marker response was influenced by the co-administration of anabolic steroids.[21] Sixty-three men and 33 women aged 18–40 years were studied. All subjects received either GH (2 mg/day) or placebo by subcutaneous injection for 8 weeks. Men also received intramuscular testosterone (250 mg/week) or placebo for 5 weeks. Consistent with the findings of the GH-2000 project, the administration of rhGH led to significant increases in IGF axis and collagen markers that were greater in men than women. Insulin-like growth factor-I showed the greatest increase of the IGF axis confirming the value of IGF-I identified by the GH-2000 team. Interestingly, the relative incremental responses of the collagen markers were greater than the IGF markers, especially for P-III-P. The collagen markers increased and decreased more slowly with most remaining elevated after six weeks. Testosterone was found to amplify the response of P-III-P to GH by more than 1.5-fold but did not affect any other marker. When given alone, testosterone had no significant effect on IGF-I but led to a 70% increase in P-III-P, which alone would be insufficient to create a false positive.

The GH-2004 project has undertaken a further rhGH administration study in subjects of non-White European ethnicity.[39] In total, 31 male and 13 female amateur athletes of Indo-Asian, Afro-Caribbean or Oriental ethnic origin were randomly assigned to receive placebo, low dose (0.1 IU/Kg/day) or high dose (0.2 IU/Kg/day) rhGH for up to 28 days with follow up for a further eight weeks using a protocol that was similar to the original GH-2000 study. A full analysis of this study has not yet been completed but the provisional results show that both IGF-I and P-III-P rise following GH administration with a similar magnitude to the GH-2000 subjects suggesting that there is no major difference in response to GH between ethnicities.

Development of the Discriminant Function Formulae

The procedure used to generate the discriminant functions involves splitting the available data into two; a 'training' set of data is used to calculate the discriminant function and a 'confirmatory' set is then used to validate the sensitivity and specificity of the discriminant function.[12] The confirmatory set is required to ensure the model is applicable to the general population and not just the 'training' set.

As there are differences in the baseline and post-GH concentrations of IGF-I and P-III-P, separate discriminant functions were calculated for men and women. These were based on the logarithmic values of P-III-P and IGF-I and included a function for age to reflect the decline in GH and marker concentrations with age.

$$\begin{aligned}\text{Male score} &= -6.586 + 2.905 \times \log(\text{P-III-P}) \\ &\quad + 2.100 \times \log(\text{IGF-I}) - 101.737/\text{age} \\ \text{Female score} &= -8.459 + 2.454 \times \log(\text{P-III-P}) \\ &\quad + 2.195 \times \log(\text{IGF-I}) - 73.666/\text{age}\end{aligned}$$

The optimal discriminant functions defined by the GH-2000 group and were calibrated against the GH-2000 elite athlete population values using the relevant gender group so that each was defined as having a mean of 0 and standard deviation of 1. The formulae include a function for age to reflect the decline in GH and marker concentrations with age.

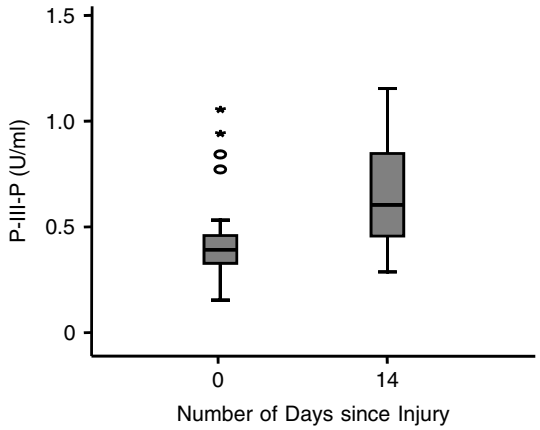

Figure 4. Change in P-III-P following a soft tissue injury after 14 days. The box plot represents the mean and 25th centiles. The whisker is the 95th centile. Outliers are shown individually.

The sensitivity of any test is inversely related to its specificity. Standard medical practice accepts as 'normal' those values that lie within two standard deviations of the mean but by definition 5% of the population are outside the 'normal range'. This would create an unacceptably high false–positive rate if applied to athletes. A cutoff point for these GH-detection formulae has not yet been agreed by the World Anti-Doping Agency but it has been suggested that a possible cutoff point at the value of 3.7 would be acceptable. Assuming the data are normally distributed, this would equate to a false positive rate of approximately 1 in 10 000 tests. Despite this stringent specificity, the test was able to identify athletes receiving GH correctly with reasonable sensitivity up to two weeks after the last GH injection, albeit with lower sensitivity in women.[12]

Effect of Injury

As P-III-P is produced in soft tissues and increases after bony fracture,[33,34] there were concerns that skeletal injury may affect the performance of the test. The GH-2004 study examined the effect of bony and soft tissue injury in 143 men and 40 women. Although there was no change in IGF-I over the 12-week follow up, P-III-P rose by approximately 40% reaching a peak 14 days after a soft tissue injury and 28–42 days after a bony injury depending on the severity (Figure 4).[40] This, however, did not cause any false positive readings in the proposed test.

Effect of Exercise

Several studies have examined the effect of exercise on IGF-I, including the GH-2000 project. Subjects underwent a maximal exercise test, following which IGF-I and P-III-P rose 20% and 5% respectively (Table 1).[27,28] Although statistically significant, this rise is much smaller than the 300% increase in the markers following the administration of GH and did not affect the performance of the test.

Independent Validation of GH-2000 Test

Ideally, further validation of the discriminant function analyses is needed using independent data sets. The GH-2000 score has been applied to the Kreischa Institut fur Dopinganalytik und Sportbiochemie GH administration.[38] Direct comparison was not possible because different assays had been used between the studies. After adjustment for assays, however, the GH-2000 score performed well, identifying 9 of the 10 athletes who had received rhGH despite the dose being lower and the duration shorter in the Kreischa study.[41]

Future Work

Despite the recognition of the need for IOC/WADA owned immunoassays in Rome in 1999, this milestone has not been reached. After the Rome workshop, the Institute for Bioanalytics (IBA) (Connecticut, USA) was successful in obtaining funding from the US Anti-Doping Agency to develop two 'in-house' immunoassays for both IGF-I and P-III-P. Unfortunately this work has not been completed.

As was predicted in Rome, there have been changes in the commercially available assays used in the GH-2000 project. The CIS P-III-P assay has been updated by the manufacturers, whereas the Nichols IGF-I radioimmunoassay was withdrawn from the market. Alternative commercial assays are available but as there is no international reference preparation for P-III-P and the one used for IGF-I has been challenged as unsuitable, the actual numerical values obtained from different assays on the same samples tend to be different. In order to interpret the results from these new assays, it is important therefore to understand how the assays relate to those from another.

The approach taken by the GH-2004 team has been to align or calibrate the new assays with the 'old' ones.[39] Where possible, samples have been measured by multiple assays. These studies have shown strong correlations between various commercial assays with differences in the potency of the standards explaining the numerical differences obtained. However, before the test can be fully implemented, further samples are currently being obtained from elite athletes to confirm that this approach is valid.

Although at present the marker approach used by the GH-2004 team relies on the use of immunoassays, there has been work to develop mass spectrometry methods for IGF-I.[42,43] The first method developed by Bredehoft and colleagues is based on immunoaffinity isolation and purification followed by liquid chromatography and electrospray ionization tandem mass spectrometry.[42] The diagnostic product ions were characterized using an Orbitrap mass spectrometer with high resolution and high accuracy properties and employed for triple quadrupole MS/MS analysis. This method has provided lower limits of detection (LLODs) between 20 and 50 ng/mL and a precision of $<$15% at the LLOD as well as at higher concentration levels. There was a reasonable correlation with values obtained from a commercially available immunoradiometric assay but the absolute concentrations were lower for the mass spectrometry procedure.

The second method is based on the reduction and alkylation of cysteine residues followed by tryptic digestion. The samples are then analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).[43] The working concentration ranges are 2–8 ng/µl for IGF-1 with reasonable reproducibility. The same group have also developed a method for IGFBP-3.

Conclusion

Growth hormone is widely abused by athletes seeking to exploit its anabolic and lipolytic properties. Over the last decade, there have been major advances in methodologies to detect GH and this should mean that before long athletes will not be able to take GH with impunity.

Acknowledgements

The author is the co-principal investigator of the GH-2004 project, which is funded by the World Anti-Doping Agency and US Anti-Doping Agency. I would like to acknowledge the rest of the GH-2004 team. I would like to thank all the athletes, both amateur and professional, who volunteered to take part in this study. I thank the nurses of the Wellcome Trust Clinical Research Facility at the University of Southampton for their assistance in recruitment and follow-up of the GH-2004 subjects. The GH-2004 team is indebted to the GH-2000 team who left us a legacy of invaluable samples.

References

[1] R. I. Holt, P. H. Sonksen, *Br. J. Pharmacol.* **2008**, *154*(3), 542.
[2] World Anti-Doping Agency, The World Anti-Doping Code – The 2008 Prohibited List International Standard, http://www.wada-ama.org/rtecontent/document/2008_List_En.pdf, accessed 5 August 2009.
[3] P. N. Prinz, E. D. Weitzman, G. R. Cunningham, I. Karacan, *J. Gerontol.* **1983**, *38*(5), 519.
[4] R. Savine, P. Sonksen, *Horm. Res.* **2000**, *53*(Suppl 3), 37.
[5] C. H. Albini, T. Quattrin, R. L. Vandlen, M. H. MacGillivray, *Pediatr. Res.* **1988**, *23*(1), 89.
[6] J. R. McCormick, P. H. Sonksen, J. S. Soeldner, R. H. Egdahl, *Surgery* **1969**, *66*(1), 175.
[7] D. Owens, M. C. Srivastava, C. V. Tompkins, J. D. Nabarro, P. H. Sonksen, *Eur. J. Clin. Invest.* **1973**, *3*(4), 284.
[8] D. E. Flanagan, M. C. Taylor, V. Parfitt, R. Mardell, P. J. Wood, B. A. Leatherdale, *Clin. Endocrinol.* **1997**, *46*(4), 425.
[9] C. M. McHugh, R. T. Park, P. H. Sonksen, R. I. Holt, *Clin. Chem.* **2005**, *51*(9), 1587.
[10] M. Bidlingmaier, Z. Wu, C. J. Strasburger, *J. Pediatr. Endocrinol. Metab.* **2001**, *14*(8), 1077.
[11] M. Bidlingmaier, Z. Wu, C. J. Strasburger, *Bailliere's Best Pract. Res. Clin. Endocrinol. Metab.* **2000**, *14*(1), 99.
[12] J. K. Powrie, E. E. Bassett, T. Rosen, J. O. Jørgensen, R. Napoli, L. Sacca, J. S. Christiansen, B. A. Bengtsson, P. H. Sönksen; GH-2000 Project Study Group, *Growth Horm. IGF Res.* **2007**, *17*(3), 220.
[13] G. Baumann, *Horm. Res.* **1999**, *51*(Suppl 1), 2.
[14] M. Bidlingmaier, J. Suhr, A. Ernst, Z. Wu, A. Keller, C. J. Strasburger, A. Bergmann, *Clin. Chem.* **2009**, *55*(3), 445.
[15] Z. Wu, M. Bidlingmaier, R. Dall, C. J. Strasburger, *Lancet* **1999**, *353*(9156), 895.
[16] M. Irie, M. Ueki, Y. Kishikawa, M. Nishii, T. Kawahara, *Growth Horm. IGF Res.* **2009**, *19*(4), 352.
[17] R. I. Holt, *Pediatr. Endocrinol. Rev.* **2007**, *4*(3), 228.
[18] J. D. Wallace, R. C. Cuneo, M. Bidlingmaier, P. A. Lundberg, L. Carlsson, C. L. Boguszewski, J. Hay, M. Boroujerdi, A. Cittadini, R. Dall, T. Rosén, C. J. Strasburger, *J. Clin. Endocrinol. Metab.* **2001**, *86*(4), 1731.
[19] J. D. Wallace, R. C. Cuneo, M. Bidlingmaier, P. A. Lundberg, L. Carlsson, C. L. Boguszewski, J. Hay, M. L. Healy, R. Napoli, R. Dall, T. Rosén, C. J. Strasburger, *J. Clin. Endocrinol. Metab.* **2001**, *86*(1), 200.
[20] A. Keller, Z. Wu, J. Kratzsch, E. Keller, W. F. Blum, A. Kniess, R. Preiss, J. Teichert, C. J. Strasburger, M. Bidlingmaier, *Eur. J. Endocrinol.* **2007**, *156*(6), 647.
[21] A. E. Nelson, U. Meinhardt, J. L. Hansen, I. H. Walker, G. Stone, C. J. Howe, K. C. Leung, M. J. Seibel, R. C. Baxter, D. J. Handelsman, R. Kazlauskas, K. K. Ho, *J. Clin. Endocrinol. Metab.* **2008**, *93*(6), 2213.

[22] World Anti-Doping Agency, International Standard for Laboratories Version 5.0, http://www.wada-ama.org/rtecontent/document/lab_08_V_05.pdf, accessed 5 August 2009.
[23] O. Barroso, P. Schamasch, O. Rabin, *Growth. Horm. IGF Res.* **2009**, *19*(4), 369.
[24] M. G. Giannoulis, M. A. Boroujerdi, J. Powrie, R. Dall, R. Napoli, C. Ehrnborg, C. Pentecost, A. Cittadini, J. O. Jørgensen, P. H. Sonksen; GH-2000 Study Group, *Clin. Endocrinol.* **2005**, *62*(3), 315.
[25] R. H. Wu, Y. St Louis, J. DiMartino-Nardi, S. Wesoly, E. H. Sobel, B. Sherman, P. Saenger, *J. Clin. Endocrinol. Metab.* **1990**, *70*(6), 1612.
[26] S. Momomura, Y. Hashimoto, Y. Shimazaki, M. Irie, *Endocr. J.* **2000**, *47*(1), 97.
[27] J. D. Wallace, R. C. Cuneo, P. A. Lundberg, T. Rosén, J. O. Jørgensen, S. Longobardi, N. Keay, L. Sacca, J. S. Christiansen, B. A. Bengtsson, P. H. Sönksen, *J. Clin. Endocrinol. Metab.* **2000**, *85*(1), 124.
[28] J. D. Wallace, R. C. Cuneo, R. Baxter, H. Orskov, N. Keay, C. Pentecost, R. Dall, T. Rosén, J. O. Jørgensen, A. Cittadini, S. Longobardi, L. Sacca, J. S. Christiansen, B. A. Bengtsson, P. H. Sönksen, *J. Clin. Endocrinol. Metab.* **1999**, *84*(10), 3591.
[29] A. Juul, S. Møller, E. Mosfeldt-Laursen, M. H. Rasmussen, T. Scheike, S. A. Pedersen, K. W. Kastrup, H. Yu, J. Mistry, S. Rasmussen, J. Müller, J. Henriksen, N. E. Skakkebaek, *J. Clin. Endocrinol. Metab.* **1998**, *83*(12), 4408.
[30] R. Dall, S. Longobardi, C. Ehrnborg, N. Keay, T. Rosén, J. O. Jørgensen, R. C. Cuneo, M. A. Boroujerdi, A. Cittadini, R. Napoli, J. S. Christiansen, B. A. Bengtsson, L. Sacca, R. C. Baxter, E. E. Basset, P. H. Sönksen, *J. Clin. Endocrinol. Metab.* **2000**, *85*(11), 4193.
[31] O. D. Wolthers, C. Heuck, L. Heickendorff, *Clin. Chem.* **2001**, *47*(9), 1721.
[32] S. Longobardi, N. Keay, C. Ehrnborg, A. Cittadini, T. Rosén, R. Dall, M. A. Boroujerdi, E. E. Bassett, M. L. Healy, C. Pentecost, J. D. Wallace, J. Powrie, J. O. Jørgensen, L. Saccà, *J. Clin. Endocrinol. Metab.* **2000**, *85*(4), 1505.
[33] K. Haukipuro, L. Risteli, M. I. Kairaluoma, J. Risteli, *Surgery* **1990**, *107*(4), 381.
[34] S. W. Veitch, S. C. Findlay, A. J. Hamer, A. Blumsohn, R. Eastell, B. M. Ingle, *Osteoporosis Int.* **2006**, *17*(3), 364.
[35] M. L. Healy, R. Dall, J. Gibney, E. Bassett, C. Ehrnborg, C. Pentecost, T. Rosen, A. Cittadini, R. C. Baxter, P. H. Sönksen, *J. Clin. Endocrinol. Metab.* **2005**, *90*(2), 641.
[36] I. Erotokritou-Mulligan, E. E. Bassett, D. A. Cowan, C. Bartlett, C. McHugh, P. H. Sönksen, R. I. Holt; GH-2004 group, *Clin. Endocrinol.* **2009**, *70*(1), 161.
[37] A. E. Nelson, C. J. Howe, T. V. Nguyen, K. C. Leung, G. J. Trout, M. J. Seibel, R. C. Baxter, D. J. Handelsman, R. Kazlauskas, K. K. Ho, *J. Clin. Endocrinol. Metab.* **2006**, *91*(11), 4424.
[38] A. Kniess, E. Ziegler, J. Kratzsch, D. Thieme, R. K. Muller, *Anal. Bioanal. Chem.* **2003**, *376*(5), 696.
[39] R. I. Holt, E. E. Bassett, I. Erotokritou-Mulligan, C. McHugh, D. Cowan, C. Bartlett, P. H. Sönksen; GH-2004 group, *Growth Horm. IGF Res.* **2009**, *19*(4), 346.
[40] I. Erotokritou-Mulligan, E. E. Bassett, C. Bartlett, D. Cowan, C. McHugh, R. Seah, B. Curtis, V. Wells, K. Harrison, P. H. Sönksen, R. I. Holt; GH-2004 Group, *J. Clin. Endocrinol. Metab.* **2008**, *93*(7), 2760.
[41] I. Erotokritou-Mulligan, E. E. Bassett, A. Kniess, P. H. Sonksen, R. I. Holt, *Growth Horm. IGF Res.* **2007**, Oct; *17*(5), 416–423. Epub 2007 Jun 20.
[42] M. Bredehoft, W. Schanzer, M. Thevis, *Rapid Commun. Mass Spectrom.* **2008**, *22*(4), 477.
[43] S. Kirsch, J. Widart, J. Louette, J. F. Focant, P. E. De, *J. Chromatogr. A* **2007**, *1153*(1–2), 300.