GH-2000 Final Report

GH-2000 A Methodology for the Detection of Doping with Growth Hormone and Related Substances.

A Biomed 2 Project

Contract Number: BMH4 CT950678

Official Contractual period: 1st January 1996-31st January 1999

Co-ordinator: Prof. Peter Sönksen, U.M.D.S, St Thomas' Hospital, London, UK

Participants:

a. Partners:

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2. Prof. J.S. Christiansen, Aarhus University Hospital, Aarhus, Denmark
3. Prof. L. Sacca, University Federico II, Naples, Italy
4. Prince Alexander De Merode, IOC Medical Commission, Lausanne, Switzerland.
5. Dr Anne-Marie Kappelgaard, Novo Nordisk, Gentofte, Denmark.
6. Dr Lynda Fryklund, Pharmacia Upjohn, Stockholm Sweden.
7. Dr Laurent Rivier, Swiss Laboratory for Doping Analysis, Lausanne, Switzerland. (Separately funded from Swiss Office for Education & Science)

b. Scientific Collaborators:

1. Prof. Philip Brown, Drs Eryl Bassett & Mike Kenward, University of Kent, UK
2. Dr Ross Cuneo, Ms Jennifer Wallace, Princess Alexandra Hospital, Brisbane, Australia.
3. Prof. Rob Baxter, Royal North Shore Hospital, St Leonards, NSW, Australia.
4. Professor Christian Strasburger (Department of Medicine, Innenstadt Hospital of Ludwig-Maximilans University, Munich, Germany)

Number of joint publications + Patents: 12 Publications submitted to date, no patents
Another 10 planned for 1999

Part A

Title: GH-2000 A Methodology for the Detection of Doping with Growth Hormone & Related Substances

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9. Dr Ross Cuneo, Ms Jennifer Wallace, Princess Alexandra Hospital, Brisbane, Australia.
10. Prof. Rob Baxter, Royal North Shore Hospital, St Leonards, Queensland, Australia.
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Background: Growth hormone (GH) is a naturally occurring hormone which has been shown to have powerful effects to build muscle and reduce fat. These anabolic properties have been widely recognised with the result that GH is now being widely abused in sport. This misuse of GH has increased dramatically as a result of its increased availability, the lack of a test to detect its use and the effective testing program now in place to deter the use of anabolic steroids. The long-term administration of high dose GH is potentially dangerous. GH use has been banned by most international sporting bodies including the International Olympic Committee.

Objectives and Primary Approaches: The aim was to develop a methodology for the detection of the abuse of exogenously administered GH, particularly in relation to sport, and to validate this such that it would withstand legal challenge and allow conviction of those using GH illegally. We planned to achieve this in time to pilot the methodology in time for the Sydney Olympics. The development of a test for GH abuse presents some unique problems. Unlike anabolic steroids GH is a naturally occurring substance. The blood levels of GH vary markedly with time and in response to a variety of stimuli including exercise. Very little GH appears in the urine. To measure GH concentrations alone in blood or urine is unlikely to provide a useful test. Many approaches to the problem were considered. GH does have widespread "downstream effects" to influence the blood levels of other substances which we postulated would provide a much better opportunity to develop a test of GH abuse. Relatively little is known about the physiology of these potential markers in relation to sport and "elite" athletes. Four major unique studies were carried out by the GH-2000 team. These included investigation of the physiology of GH-dependent markers in response to the administration of GH or placebo (inactive injection) to over 100 men and women. Blood samples were obtained from over 700 competing athletes to identify the stability of these markers and determine post-competition reference ranges.

Results and Discussion: The results of these studies provide much unique data on GH-dependent markers. The most useful markers of GH abuse were identified as: blood levels of insulin-like growth factor I (IGF-I), acid labile sub-unit, IGF-I binding protein 3, IGF-I binding protein 2, osteocalcin, C-terminal propeptide of type I procollagen, procollagen type III, type I collagen telopeptide. Analysis of the data from the therapeutic study of GH administration showed that combinations of GH-sensitive markers could identify GH administration much better than any individual marker. Formulae were devised to optimise the discrimination of those treated with GH. The optimal combinations of markers differed between men and women resulting in a gender specific formula. These tests were calibrated for "elite" athletes using the huge amount of post-competition data. Adjustments were made for age which had a significant effect of some of the markers used. Cut-off values for these formulae, above which it would be safe to conclude that doping with GH had occurred were made. This was done with an overriding consideration of the need to avoid false positive results. The cut-off values chosen will allow the determination of GH abuse with a false positive rate of around 1 in 300,000.

Major Scientific Breakthroughs and Industrial Applications: This project has developed a test for the detection of illegal GH administration. It has also described a wealth of new information regarding the physiology of GH-dependent markers in "elite" athletes and in relation to exercise and GH administration.

No. of Publications: Seven abstracts have already been published and 5 have been submitted to the American Endocrine Society. One manuscript has been submitted to the Journal of Clinical Investigation and 6 manuscripts are in preparation.

Major Cooperative Links: This study has resulted in the bringing together of most of the European expertise in GH physiology and cooperation with others world-wide as can be seen from the participants detailed above. It has also enhanced the relationship between academia, sports medicine and industry with consequent production of an enormous amount of unique physiological information. These links will continue to be forged and will benefit future projects.

Part B

1. Background Information:

Growth hormone (GH) is a naturally occurring protein hormone produced by the pituitary gland. It has an effect to increase longitudinal growth in children but has also been shown to have important effects in adults. Adult patients with pituitary disease who lack growth hormone have reduced muscle mass, impaired exercise performance and increased body fat. Treatment with GH replacement therapy reverses these abnormalities as well as improving exercise capacity and psychological wellbeing.

This powerful anabolic action of GH is similar to that seen with testosterone and anabolic steroids but GH acts by a separate and synergistic mechanism to these other substances. These properties have been widely recognised with the result that GH abuse has become increasingly prevalent amongst those who would seek to enhance their physical appearance and performance, particularly in the sporting arena.

This abuse is of concern, not only because of the undesirable effects on the ethics of sport and unfair competition which ensues but also because the long-term use of high doses of GH is likely to have undesirable and potentially life threatening side effects. Acromegaly is a condition whereby a pituitary tumour secretes increased amounts of GH. In the long term GH-abuse may result in cardiovascular and respiratory abnormalities including premature death, osteoarthritis and an increased risk of malignant disease. GH administered in supraphysiological doses over a similar time period may also be associated with such problems.

GH is banned by the International Olympic Committee (IOC) and most international sporting bodies but its use continues as there have, until now, been no effective way of identifying those who are cheating by this means. GH has allegedly been abused in sport for as long as 15 years without any disqualification being made as a result of its abuse. The potency and 'invulnerability' of GH are well known amongst most athletes and their trainers. This, along with the increasing availability of GH, effective testing for the detection of anabolic steroids and the increased "commercial" pressures in sport, have had the inevitable consequence of an increase in GH abuse. Anecdotal evidence such as the discovery of large quantities of GH during the Tour de France 1998 cycling competition and the World Swimming Championships in Perth in 1998 have highlighted the extent of this abuse. There are also some worrying reports from the USA suggesting a high incidence of GH use amongst high school students seeking to improve physical appearance and sporting performance. This abuse of GH has emphasised the need to develop an effective test to detect and ban athletes using this substance in order to maintain the ethos of sporting competition and deter others from using such substances.

The GH-2000 project has brought together a team of international experts in the field who have focused their research efforts on solving the problem. This project has been successful in developing a test which will detect the administration of injected (exogenous) GH which is scientifically sound and which we believe should be sufficiently rigorous to meet the demanding legal issues involved.

2. Objectives and Primary Approaches

The *a priori* declared aims of the project were:

1 To develop a methodology for the detection of use and abuse of exogenously administered growth hormone and related substances, particularly in relation to sport.
2 To develop a validation methodology sufficiently robust to withstand legal challenge and allow prosecution and conviction of those illegally using growth hormone.
3 To develop the ethical and legal framework to control and deal effectively with growth hormone abuse.
4 To have achieved these objectives in time to pilot the methodology in the Olympic Games in Australia in the year 2000.

The development of a test for GH abuse presents some unique problems. Unlike anabolic steroids and other synthetic substances, GH is a naturally occurring substance. Genetically engineered GH has an identical amino acid sequence to the majority of the naturally occurring hormone circulating in the body and so it is not possible to distinguish this by chemical analysis or immunological assay. Another major problem is that GH is secreted in pulses by the pituitary gland in response to a variety of stimuli including stress and exercise. The blood levels of GH therefore vary widely with time. In addition very little GH appears in the urine. These factors mean that simple measurement of serum or urine GH would not be able to provide a robust test for exogenous GH administration.

GH does however have widespread effects to influence the blood levels of other substances which are theoretically much more likely to provide useful marker or GH abuse (*See Appendix I for diagram of GH / IGF-I / Bone Marker Axis*). Thus GH influences the levels of another peptide called insulin-like growth factor I (IGF-I). IGF-I is transported in the blood by a number of binding proteins (IGFBPs), some of which are influenced by prevailing GH concentrations. GH also has an effect on bone formation and re-modelling that is reflected by various peptides released from the bone into the blood stream. These 'markers' of the 'downstream effects' of GH vary much less dramatically with time or in response to exercise. We postulated therefore that the measurement of these markers would provide the best opportunity of developing a test of GH abuse and so developed our methodology accordingly. Most of these markers are proteins and as such are completely degraded by the kidney. As a result, urinary excretion is too low and erratic to be of use in dope testing. A few are glycoproteins (some of the bone markers) and the pattern of their metabolism and clearance in the urine may be more favourable.

Relatively little is known about the response of many of these potential markers of GH abuse to exercise and other physiological stimuli. There is even less information about how they might vary in elite athletes. A major part of the study therefore consisted of the determination of the variation of these markers in response to a wide range of physiological circumstances.

In order to meet our aims and overcome the inherent problems, four major studies were planned and undertaken by the partnership over the 3 years:

1. **The Washout Study:** This was a relatively small preliminary double-blind placebo-controlled study of one week's rhGH administration to 17 male aerobically trained individuals (*not* competing athletes). The primary purpose of this study was aimed to examine individually and in combination, the effects of acute exercise and rhGH administration on the various potential markers with a view to determining a 'short-list' of those that are sensitive to exogenous rhGH administration but insensitive to the effects of acute

exercise. These would then be studied further and in more detail in order to develop a robust test of GH abuse.

2. **The Double-Blind (Doping Simulation) Study**: The purpose of this study was to simulate the scenario of GH doping and to develop a method of detecting those who were given rhGH injections without anyone being biased by knowing who had active treatment or 'dummy' (placebo) injections. For this 103 male and female healthy fit volunteers from four countries participated in a classical 'double-blind placebo-controlled' study. These volunteers were not competing athletes but were exercise enthusiasts and already participating in at least 2 regular training sessions per week. Each subject was randomised to receive either 0.1 or 0.2 IU/kg/day of recombinant GH or placebo for 28 days from day 0 to day 28. GH markers were studied in blood and urine before, during and for 56 days after the GH-treatment period.

3. **The Cross-sectional Study**: Blood samples were collected immediately 'post-event' from more than 700 elite athletes participating in a wide range of major National or International sporting events. The purpose of this study was to determine a '*normal*', '*post-competition*', '*reference range*' for the various markers in such highly trained individuals. It also examined the effects of the 'type of event' as well as important 'demographic factors' such as age, weight, height, body mass index, gender and ethnic group.

4. **The Longitudinal Study**: The purpose of this study was to investigate the variation that occurs in the various potential markers of GH administration within individual elite athletes at different time points in their training regimes and in response to competition and a standardised exercise test. Blood and urine samples were obtained from a sub-set of 150 elite athletes who had volunteered for the Cross Sectional Study (3. above), again from a wide range of sporting disciplines. Each athlete was followed longitudinally during one season and provided several resting samples, one post-competition sample and samples during a laboratory VO_2-max. test. This study also allows the investigation of the influence of type of sport and demographic effects on the 'stability' of the markers over time.

We also addressed a number of other potential approaches to detecting rhGH abuse

The project was carried out according to ethics standards set in the agreement of Helsinki. The GH-2000 project and all its protocols, received Ethics Committee approval in each of the four participating countries. Research team members recruited the volunteers and after appropriate briefing, every volunteer signed an individual written consent form. The research team collected the blood and urine samples and demographic details from each volunteer and these were recorded on a specially designed form for subsequent entry into the database. Blood samples were collected and centrifuged without delay and the plasma samples aliquotted into 1ml samples that were identified by a unique bar code. Samples of plasma and urine were frozen without delay and transported to base on dry-ice and thereafter stored at -70°C until sent for analysis. The identity of each sample was identified with a unique bar code, the key to which was held on the central database. It was not possible to identify from whom the sample came without reference to the database. All samples were analysed without knowledge of any details about their source.

Growth hormone and matching placebo was provided by Novo Nordisk (Denmark) and Pharmacia / Upjohn (Sweden).

Samples were dispatched to the three laboratories used by the project (deep-frozen) by professional courier. All communication of results was carried out using the bar codes and the identity of the athlete from whom the samples came was not known to the laboratories. The results returned to the co-ordinating centre were scrutinised and checked and entered into the database, electronically wherever possible. The database entry was re-checked by independent team members to minimise transcription errors. The database was built and maintained by Dr Massoud Boroujerdi and held at St Thomas' Hospital. The collection and handling of samples and demographic details was under the supervision of the Project Manager – Ms Claire Pentecost.

The three laboratories used by the project were selected through a tendering process. Each was an accredited laboratory with the desired analytical methods already established. Each had its own quality control procedures. The three laboratories used were:

1. Dr Per Arne Lundberg (Gothenberg, Sweden), Insulin-like growth factor-I (IGF-I), Osteocalcin, Procollagen type III (P-III-P), C-terminal propeptide of type I procollagen (PICP), Type I collagen telopeptide (ICTP)
2. Professor Robert Baxter (Sydney, Australia), IGF binding protein-2 (IGFBP-2), IGF binding protein-3 (IGFBP-3) & acid-labile subunit of IGFBP-3 (ALS))
3. Dr Laurent Rivier (Lausanne, Switzerland), Urinary concentrations of: hGH, IGF BP3, Pyridinium Crosslinks (Pcd) & Deoxypyridinoline Crosslinks (Dpd)

Statistical analysis was supervised and undertaken by Professor Philip Brown, Dr Eryl Bassett & Dr Mike Kenward and colleagues at the University of Kent. Further experimental statistical and biological modelling was undertaken by Professor Philip Brown and Dr Massoud Boroujerdi respectively.

The Statistics Unit at the University of Kent in collaboration with the Project Manager was in charge of resolving any data quality issues that arose through missing samples or ambiguity in any results.

Dr Ross Cuneo & Ms Jennifer Wallace joined the project as 'Scientific Collaborators' while they were undertaking a Sabbatical Year at St Thomas' Hospital. Their salaries were provided by the University of Queensland (Australia). They contributed to the design of the overall project and they were mainly responsible for designing and executing the 'Washout Study'.

Dr Laurent Rivier (IOC Laboratory, Lausanne, Switzerland) joined the project as a 'Scientific Collaborator' (funded by the Swiss Research Council) and he and his team undertook all the urine analyses.

Dr Christian Strasburger (Munich, Germany) joined the project as a 'Scientific Collaborator' (funded by the Bundesinstitut fur Sportwissenschaft (BISp), Cologne, Germany – WF 0408/08/02/97). He performed a pilot study with his own novel method of detecting recombinant human growth hormone (rhGH) using a combination of monoclonal antibody techniques.

3. Results and Discussion

3.1 Washout Study

The results of this study gave new insight into the behaviour of the various potential markers in response to exercise and a short course of rhGH. This study was particularly helpful in allowing us to identify a sub-set of markers that were sensitive to treatment with rhGH but relatively unaltered by acute exercise. These then were likely to provide the most promising markers for further detailed study. These were in two basic groups, the first arising from GH effect on the hepatic production of IGF-I and some of its binding proteins:

1. Insulin-like Growth Factor I (IGF-I)
2. IGF-I Binding Protein 3 Acid Labile Sub-unit (ALS)

3. IGF-I Binding Protein 3 (BP3)
4. IGF-I Binding Protein 2 (BP2)

The second group was those produced as a result of GH action on bone and the production of a series of GH-dependent markers as part of the bone re-modelling process activated by GH:

1. Osteocalcin
2. C-terminal propeptide of type I procollagen (PICP)
3. Procollagen type III (P-III-P)
4. Type I collagen telopeptide (ICTP)

The first set of results of this study have been submitted for publication in the Journal of Clinical Investigation. More details of this project and some of its results are given in the submitted manuscript, a copy of which is attached as Appendix II.

3.2 The "Double-Blind" (Doping Simulation) Study

This was the pivotal study of the GH-2000 project. In this more than 100 healthy, fit, 'recreational' athletes from 4 European Nations volunteered to take daily injections of either GH or a placebo ("dummy") for a month and to have blood and urine samples collected before, during and for 56 days after. They also participated in a formal 'exercise stress-test' on several occasions where blood and urine samples were collected. The results showed that those treated with growth hormone had blood levels of GH-dependent markers that clearly indicated that they had received growth hormone. More details of the analysis of these results will be reported in section 3.5 detailing the statistical methodology but in summary, the sensitivity of the selected discriminating 'GH-dependent markers' was such that it was possible to identify those taking GH. There was a marked gender difference in that detection of growth hormone use was generally easier in men than women. This appears to be due to a genetic insensitivity of young women to the effects of growth hormone. It was possible to detect the effects of growth hormone administration for as long as three months after the last dose in some people. Illustrative data from IGF-I levels are given below in **Figure 1.**

Amongst other things, it can be seen that although IGF-I peaks at day 28, some values actually fall between day 21 & day 28 raising the issue of non-compliance' with therapy.

3.3 The Cross Sectional Study

This showed that it was feasible to obtain blood samples quickly and safely from volunteer elite athletes immediately after a major national or international event. By measuring the concentrations of the potential markers of GH abuse in 'normal volunteer elite athletes', a preliminary 'reference range' for these markers has been established. The values obtained from elite athletes were (as anticipated) not all within conventional 'reference ranges' as is illustrated in **Figure 2**, confirming the need for a special reference range for elite athletes.

Much of the apparent between-sport variability observed was attributable to the fact that markers are age and gender dependent. Most markers apart from BP2 decline with age. The variance of values about the mean, for markers within this population of elite athletes varied from 20% for IGF BP-3 to nearer 40% for IGF BP-2 and osteocalcin. The results will be published in detail in the scientific papers.

3.4 The Longitudinal Study

This study involved a sub-set of elite athletes who volunteered for the **Cross-sectional Study** and who were prepared to provide additional samples ‘in’ and ‘out’ of competition & training over a 12 month period. This enabled us to establish the variability and pattern of response of these potential ‘markers’ of rhGH abuse to training, events, de-training, season and other life-style changes. Volunteers also undertook a ‘laboratory-based sport-specific maximal effort test’ with measurement of the various markers in blood and urine. The results of this study (still under analysis and not presented in detail here), confirm the relative stability of these ‘markers’ with time and the likely genetic-determination of blood ‘marker’ concentrations.

Additional data on the stability of markers with time comes from the placebo arms of both the Washout and Double-Blind study where data up to three months is available.

A summary table of the analysis of these results is given below: It can be seen that the ‘within-athlete’ coefficient of variation is remarkably low for most of the markers. It is known that some of this apparent variability appears greater than the true biological variability as for instance, with osteocalcin there was some abnormally low values as a result of haemolysis.

Marker	Sex	Central value	Coefficient of Variation
ALS	M	225	10%
ALS	F	286	11%
BP2	M	369	23%
BP2	F	357	26%
BP3	M	3.80	12%
BP3	F	4.40	12%
ICTP	M	4.02	13%
ICTP	F	4.03	15%
IGF-I	M	259	13%
IGF-I	F	330	15%
OST	M	11.33	9%
OST	F	9.57	13%
PIIIP	M	0.489	11%
PIIIP	F	0.504	14%
PICP	M	159	10%
PICP	F	132	12%

Central Value is an estimate of the median level for a given marker. Coefficient of variation is a measure of the ‘within-athlete’ variability of that marker over time, as best estimated from ‘Double-Blind’ study

3.5 Use of the Data to Construct a Doping Test

3.5.1 Background

The principles underlying the derivation of a doping test were these. Since the double-blind trial was tightly controlled, it was suitable for substantial multivariate statistical analyses to be conducted. These analyses showed that combinations of GH-sensitive markers could distinguish between GH active and placebo groups much better than individual markers could. See, for example **Figure 3** below:

The multivariate techniques led to several possible combinations of GH-sensitive markers, which discriminated well between the GH active and placebo groups. Because the patterns of response to GH administration differ between males and females, separate discrimination functions were devised for the two sexes.

The general levels of GH-sensitive markers were found to differ between the `healthy volunteers' used in the double-blind trial and the `elite athletes' for whom the test needs to be calibrated. (See, for example, **Figure 2** which shows the distributions of P-III-P levels in placebo group males in the double-blind trial, and for the male athletes studied in the cross-sectional trial.).

To validate a test for use in high-level competition, a cut-off value should be calculated on the basis of the distribution found for the relevant function in the cross-sectional trial. It is well known, however, that many GH-sensitive markers are highly dependent on factors such as age. See, for example, **Figure 4**, showing the dependence of P-III-P on age, for males in the cross-sectional trial.
When settling on appropriate cut-off values for use in high-level competitions, one therefore needs to work with age-adjusted values.

3.5.2 Combining the Markers from the Double Blind Study

Statistical techniques used in devising suitable marker combinations included discriminant analysis, binary and ordinal logistic regression, as well as many univariate and multivariate diagnostic and model-building methods. From distributional considerations, and in line with best statistical practice, all markers were expressed on a logarithmic scale; this improved the performance of the analyses and is illustrated for plasma IGF-I in **Figure 1**.

The time trace shown in **Figure 1** confirms, as one would expect, that doping should be easier to detect during (21 or 28 days) or shortly after (30 or 33 days) administration of the drug. Approaches to developing marker combinations involving the entire trace are still under investigation. However, the evidence from the Tour de France, mentioned earlier, suggests that athletes may well continue to take GH very close to the start of competition. If this is so, a method of discrimination based mainly on the time points at which the traces of the different groups are most different might then be justifiable, at least in the first instance.

The work reported here therefore concentrates attention on the 21-day figures. With the markers expressed on a logarithmic scale, combinations which seemed particularly promising involved mainly markers P-III-P and IGF-I. For example, a combination which worked particularly well for males was

$$EM1 = -2.269 + 0.7207 * p3p + 0.5210 * igf$$

where p3p and igf scores are the logarithms of p3p and igf scores respectively. A similar combination which worked well for females was EF3, defined as

$$EF3 = -4.973 + 1.1317 * p3p + 1.0125 * igf$$

In both cases, the larger the score obtained, the more likely it is that the athlete was in a GH group rather than the placebo group.

Figures 5 and 6: show the distribution of values obtained on these scores during the double-blind trial. The bottom line in each figure shows the distribution of all `clean' scores (all visits for subjects in the placebo group, plus the 'day 0' measurement, before the start of treatment, for those in the GH groups). The scaling on the horizontal axis is such that these day 0' scores have mean 0 and standard deviation 1.

In Figures 5 and 6, each line above the bottom one shows the range of values of subjects in the two GH groups on the day concerned. It can be seen that, for males, discrimination is virtually perfect for days 21-30, and that discriminating power slowly decays thereafter. The results for females are not as clear-cut, but still show substantial discriminating power for some time after GH treatment stops at 28 days.

Tests of differing specificity result from different cut-off values for EM1 and EF3. A cut-off value of 3.5 (3.7, 4.0, 4.5) will give a specificity of 1 in 5000 (10000, 32000, 340000). Taking a cut-off value of 4.0 (less than 1 in 32,000 chance of being wrong), so as to produce very good specificity, it can be seen from Figure 5 that 22 out of the 28 male subjects on the active drug would be detected on day 21, a sensitivity of about 79%. The corresponding figures for females, from Figure 6, are 8 out of 27, a sensitivity of about 30%.

Looking at various levels of probability of wrongly allocating a placebo-treated athlete to rhGH, the following table is derived from the information in **Figures 5 & 6:**

Cut-off value (EM1 or EF3	Approximate Specificity of test	Success rate on: Day 21 Males	Success rate on: Day 21 Females	Success rate on: Day 28 Males	Success rate on: Day 28 Females	Success rate on:Day 30 Males	Success rate on: Day 30 Females
3.5	**1 in 5000**	**23/28**	**11/27**	**24/28**	**10/26**	**15/24**	**8/27**
3.7	**1 in 10,000**	**23/28**	**9/27**	**24/28**	**10/26**	**13/24**	**7/27**
4.0	**1 in 32,000**	**22/28**	**8/27**	**24/28**	**9/26**	**12/24**	**5/27**
4.5	**1 in 340,000**	**17/28**	**5/27**	**19/28**	**6/26**	**7/24**	**3/27**

3.5.3 Calibration Using the Cross-Sectional Study

As noted earlier, the distribution of individual GH-sensitive markers differs between healthy volunteers and elite athletes. Calibration of a test thus requires use of the data on elite athletes contained in the cross-sectional trial.

Plots of the marker combination scores against age, for athletes in the cross-sectional trial, are shown in **Figures 7 for males and Figure 8 for females**, the dependence being very clear-cut. One must therefore adjust for age when producing a doping test criterion. For both sexes, the dependence on age is of a fairly smooth form. A simple function of derived marker EM1 and age, which removes the dependence on age is:

$$EM1b = 2.560 + 4.031 * EM1 - 101.737 / age,$$

Figure 7 Males

Figure 8 Females

where age is measured as usual in years. This age-adjusted marker has mean 0 and standard deviation 1 over the sample of elite male athletes.

The project team are still working on checking the extent of any dependence this has on an athlete's body-type or other characteristic, but any dependence seems likely to be relatively slight.

Similar calibration has been undertaken for female elite athletes, leading to the age-adjusted derived marker

EF3b = 2.322 + 2.168 * EF3 - 73.666 / age ,

which also has mean 0 and standard deviation 1 over the relevant sample.

3.5.4 Cut-off values

The best tests available at present are those based on the age-adjusted derived markers EM1b and EF3b, though work is continuing in an attempt to obtain improved discrimination. If these tests were to stand, the final question to be answered concerns the level of EM1b or EF3b at which it would be safe to conclude that artificial rhGH had been administered. There is, of course, an overriding need to avoid false positives. Examination of the distributions of EM1b and EF3b over the relevant samples shows that both are extremely close to the standardised normal distribution. *For this distribution, only about 1 observation in 32000 exceeds a value of 4, while the proportion exceeding a value of 4.5 is less than 1 in 300000. A cut-off value of 4.5 for EM1b (males) or EF3b (females) should therefore be extremely safe.*

3.6 Alternative Modelling Strategy Using the Trajectory of Markers Through Time in the Double Blind Study

This method was developed as an alternative means of analysing the double blind study using *all* the time points for each individual.

Blood Marker data on an individual are available generally at seven time points, at day zero, and at days 21, 28, 30, 33, 42, 84. It is desirable to model the behaviour of the markers simultaneously for each individual so as to:

(1) Improve the estimation of covariability of markers as they change over time.

(2) Predict with greater accuracy whether a new individual with one time presentation fits somewhere along the curve.

(3) Ascertain through a probability distribution when a presented individual last took growth hormone.

The model was curve plus error with a different curve depending on the dose group of the individual and also different for males and females. The curves were just estimated at the sampled time points. We adopted a Bayesian analysis with multivariate normal error structure (after transformation) with one term for covaribility of markers and and one covariability matrix across time.

The results for the two markers P-III-P and IGF-I were good but with all eight markers included they were uniformly better. For a rhGH-dosed individual in the test sample (not used for estimation) sampled in the interval 21-33 days, there was always at least one presentation time when the probability of the individual being incorrectly classified (as placebo) was less than the cut-off of 1 in 10,000 and often substantially less, allowing a margin of error for uncertainty in the estimation process.

As an example, take the Danish male number 15 from the test set of 10 males. This man was on low-dose growth hormone up to day 28. The table of posterior probabilities over the diagnosis drugged or not drugged by presentation time is:

Table indicating the probability of doping for an individual taking GH in the double-blind study according to day of sampling. GH administered daily from day 1 up to and including day 28.

	Baseline	*Day 21*	*Day 28*	*Day 30*	*Day 42*	*Day 84*
Placebo	0.4	$3e^{-8}$	$9e^{-9}$	$3e^{-9}$	0.007	0.6
GH treated	0.6	1- ($3e^{-8}$)	1- ($9e^{-9}$)	1- ($3e^{-9}$)	0.993	0.4

Here '$3e^{-8}$' signifies '3 times 10 to the power of minus 8', a very small probability in comparison with '$1e^{-4}$' or in it's other notation, 'a probability of less than 1 in 10,000'. Day 0 is before the man takes growth hormone and the markers have reverted to their placebo levels by day 84, but there is still strong suspicion of doping at day 42 two weeks after last taking growth hormone. There are also calculated probability distributions over the time in dosing history that the individual presents, and these will be given in the future publication of this work in the statistical literature. It involves sophisticated statistical modelling which to our knowledge has not been attempted before. A particular strength of this approach is that it gains power by using simultaneous data from all the GH-dependent markers (*PHIL – IS THIS CORRECT?).*

3.7 Other Potential GH Detection Methodologies

3.7.1 Detection of Urinary Markers

Urine samples taken from the double-blind study of GH administration were analysed for creatinine (to assess and allow correction for the degree of urine concentration), GH and IGF-BP3 and for the bone markers that are excreted in the urine: pyridinium (Pcd) and deoxypyridinoline (Dpd) crosslinks. As expected the concentrations of the peptides GH and IGF-BP3 were very low (they are most-likely directly metabolised by the kidney and broken down into constituent amino acids), showed much variation and showed only very weak discriminating powers between the GH-treated and placebo-treated groups at any time point. The concentrations of the metabolites of the bone markers, Pyridinium and deoxypyridinoline crosslinks, showed a significant increase with GH treatment. In very preliminary analyses, the results from these two metabolites of the bone markers showed encouraging powers of discrimination. *The results of these urinary markers only became available shortly before this report was written and further statistical work needs to be carried before their role can be ascertained. It is possible that some aspect of this might provide a useful adjunct to the blood testing.*

3.7.2 Direct Detection of GH Isoforms

The pituitary gland normally produces different isoforms of GH. Biosynthetic rhGH is however, all of the one 22kDa isoform and when administered seems to suppress the pituitary-derived non 22kDa, at least post exercise. Measurement of the relative concentrations of 22 and non-22kDa isoforms represents a potential method for detecting GH abuse. It has, in the past been difficult to distinguish between these various isoforms. Dr Christian Strasburger (under sponsorship of the Bundesinstitut für Sportwissenschaft (BISp)) has however developed new assays to look at this aspect. He has collaborated with GH-2000 and analysed all the samples from the Washout Study using his technique. Details of this method and results are given in more detail in Appendix III. Using his unique and patented method, Dr Strasburger was convincingly able to detect all but one of the volunteers taking rhGH in the Washout Study and all those treated with rhGH in a further independent study. This use of this more direct technique adds another very useful dimension to the detection of GH abuse. This approach however, will be limited by the fact that it can only detect GH doping for a short time after GH administration due to the short half-life of GH in the circulation (16 minutes). In these preliminary studies the specificity and sensitivity of his methods are however promising and the method deserves further evaluation. It may be that this approach, if successful, could be used on its own or to complement the other detection methods. Unfortunately the further validation of this technique was not possible within the time-scales and budget of the current project but we feel that the results were sufficiently encouraging to put it firmly on the agenda for GH-2000 Phase 2, should this ever go ahead.

3.7.3 Detection of Antibodies to GH

Antibodies may appear in the blood as a result of the introduction of any "foreign" substance. This is rare, but can occur in response to the introduction of genetically engineered human hormones such as GH. Studies so far have however shown only extremely low concentrations (low titre and low affinity) of these antibodies in very few patients who have received rhGH. This approach was therefore ruled-out as a useful means of detecting GH abuse.

3.7.4 Detection of Antibodies to E. coli

Synthetic GH is produced using a bacterium called *E. coli*. Although the hormone is purified, some tiny amount of *E. coli* remnants may remain resulting in the production of antibodies to these after administration. *E. coli* are however universal and all human subjects have antibodies to *E. coli* already present making this an useless detection methodology.

3.7.5 Detection of GH Degradation Products

Synthetic GH degrades while in storage and the possibility of measuring these degradation products has been suggested as a possible mechanism for detecting GH abuse. Current information suggests that this will be difficult as the degradation appears to occur extremely slowly, little is known about the kinetics of these products once they are injected and little is known about the degradation of endogenous pituitary GH.

3.7.6 Identification of Specific Synthetic DNA Sequences

The manufacture of rhGH involves the production of specific promoter sequences of DNA which are inserted into the bacterium to allow it to then manufacture GH. These manufactured plasmids are not naturally occurring. Some small residual amounts of these specific DNA fragments remain in the final GH product and can be detected in the serum of those who have received this GH. Pharmacia/Upjohn have undertaken some work looking at the possibility of being able to detect these specific DNA fragments.

The advantages of this methodology are that it is highly selective and highly sensitive being able to detect 10-100 molecules. It is however highly sensitive to contamination and requires experienced personnel and is time consuming (2 days work for 1 person for 20-30 samples). Sensitivity and specificity are a problem. Unfortunately each manufacturer has a unique expression system. The development of this method would therefore require collaboration between the 6 main GH manufacturers. There are also several new manufacturers of rhGH in Asia which might cause more difficulty. Pharmacia / Upjohn have now dropped their work on this method.

This methodology still has potential but there are still very many unknown factors. Thus, the uptake of the plasmid into the blood from a sub-cutaneous injection is not known and nor is the clearance from the circulation or whether or not these fragments appear in the urine. In addition more work needs to be done in developing an extraction method from blood and on storing and handling procedures.

1.8 Developing a Legal and Ethical Framework

Discussions took place with members of the IOC Juridical Committee at several times during the project and the preliminary results of the GH-2000 project were presented formally to a representative of the IOC Juridical Commission during the Winter Olympic games at Nagano in February 1998. In discussion with the IOC Medical Commission Sub-Commission ‘Doping and Biochemistry in Sport’, the issues concerning converting scientific probabilities into legal terminology was addressed in depth. It was agreed that this was an important issue and that it was necessary to reconcile the legal and scientific terminology and harmonise understanding as well as language. As far as GH-2000 was concerned, it was decided that this would best be left until the Final Report had been handed to the IOC. It could then be considered in detail by the Juridical Committee. In preliminary discussion it was agreed that ‘Beyond Reasonable Doubt’ roughly equated to a ‘Probability of >1 in 10,000.

Summary:

- The project went very much according to plan and completed the bulk of its work on time and within budget – further statistical analyses are however, still being undertaken and a supplementary report will be produced shortly
- We have proven beyond all reasonable doubt that it is possible to detect illegal use of recombinant human growth hormone by elite athletes and have devised tests to do so. The methodology will be verified by publication in peer-reviewed scientific journals
- The best tests devised so far, EM1b and EF3b, are scientifically sound. Applying them with a specificity of 1 in 10,000 would result in a very good chance of detecting a male athlete who was taking GH in amounts and over a period similar to that in the Double-Blind trial. The chance of detecting a female athlete would be smaller but still substantial
- The results were largely in keeping with our knowledge and understanding of the normal GH axis and much as anticipated when the project was first formulated

4. Major Scientific Breakthroughs and Industrial Applications

Our major contribution is that we have developed a method for detecting rhGH abuse, which may well be able to detect GH administration as long as 14 days previously. We have also described the normal and elite athlete’s hormonal profiles in response to exercise and major sporting events. We have in addition, developed ‘reference ranges’ for the GH-dependent markers in response to exercise and rhGH administration. All of this is new and of great medical and scientific interest. It will lead to a series of first rate publications in top scientific journals.

These results although specific for growth hormone, will also contribute very considerably to the development of test to detect doping with GH-releasing substances and IGF-I.

It is possible that new assays will emerge later as a direct result of this study but this will depend on whether or not the project is taken forward to implement a ‘Working Test’ for GH abuse.

5. Publications

1. Wallace J., Cuneo R., Baxter R., Rosén T., Bengtsson B-Å., Dall R.,, Jorgensen J-O., Orskof H., Cittadini A., Longobardi S.,, Sacca L., Pentecost C.,, Healy M-L., Sönksen P.H. Endocrine markers of growth hormone abuse in sport. Proceedings of the 41st Annual Scientific Meeting, Endocrine Society of Australia, volume 41, p.55, abstract #7

2. Ross C. Cuneo, Jennifer D. Wallace, Thord Rosén, Per-Arne Lundberg, Beng-Åke Bengtsson, R Dall, Jens O.L. Jorgensen, Jens S Christiansen, Antonio Cittadini, Salvatore Longobardi, Luigi Sacca, Claire Pentecost, Nicola Keay, Peter Sönksen. Use of bone markers to detect growth hormone abuse in sport Proceedings of the 41st Annual Scientific Meeting, Endocrine Society of Australia, Perth, August 1998, volume 41, p.55, abstract #8

3. Ross C Cuneo and Jennifer D Wallace. Doping in sport, an overview. The Clinical Biochemist Reviews 1998;19:(iii):70, Proceedings of the Australasian Association of Clinical Biochemists, 36th Annual Scientific Conference, Brisbane, August 1998

4. JD Wallace, RC Cuneo, R Baxter, T Rosén, B-Å Bengtsson, R Dall, JO Jorgensen, C Pentecost, ML Healy, A Cittadini, S Longobardi, Detection of growth hormone abuse in athletes Growth Hormone & IGF Research Vol 8:4:329, August 1998,abstract #O66 of the Growth Hormone Research Society Conference, San Francisco, 3-7 September 1998

5. JD Wallace, RC Cuneo, T Rosén, B-Å Bengtsson, P-A Lundberg, R Dall,JS. Christiansen, C Pentecost, N Keay, A Cittadini, S Longobardi, Bone Markers and Growth Hormone (GH) Abuse in Athletes Growth Hormone & IGF Research Vol 8:4:348, August 1998,abstract #P71 of the Growth Hormone Research Society Conference, San Francisco, 3-7September 1998.

6. Jennifer D Wallace and Ross C Cuneo. Endocrine markers of growth hormone abuse in sport: Invited lecture (NB same title but different content to ESA Abstract) Proceedings of the Sports Medicine Australia Conference, Perth, October 1998 (in press)

7. Jennifer D Wallace, Ross C Cuneo, Robert Baxter, Hans Ørskov, Nicola Keay, Claire Pentecost, Rolf Dall, Thord Rosén, Jens Otto Jørgensen, Antonio Cittadini, Salvatore Longobardi, Luigi Sacca, Jens Sandahl Christiansen, Bengt-Åke Bengtsson, Peter H Sönksen. Detection of Growth Hormone (GH) abuse in sport: responses of the GH and insulin-like growth factor axis to exercise, GH administration and GH withdrawal in trained adult males. Journal of Clinical Investigation submitted 1998.

The following are abstracts submitted to the American Endocrine Society for presentation in San Diego, US, June 12-15 1999.

1. S Longobardi, N Keay, C Ehrnborg, A Cittadini, T Rosén, M Healy, R Dall, Basset E, C Pentecost, JO Jorgensen, on behalf of the GH 2000 study group, Effects of growth hormone administration on bone soft tissue turnover in healthy adults and their potential usefulness in detection of growth hormone abuse/ Growth hormone (GH) effects on bone and collagen turnover in healthy adults and its potential usefulness in the detection of GH sport abuse: A double blind, placebo-controlled study.

2. M.L. Healey, R Dall, C Ehrnborg, S Longobardi, A Cittadini, JS Christiansen, C Pentecost, E Basset, Boroujerdi MA, R Baxter, T Rosén on behalf of the GH 2000 study group, GH/IGF-1 and related parameters in elite athletes-a post competition, cross-sectional study.

3. C Ehrnborg, K Lange, R Baxter, R Dall, T Rosén, B-Å Bengtsson, JS Christiansen, P Sonksen, L Sacca on behalf of the GH study group, GH/IGF-1 and related parameters in elite athletes-A longitudinal study.

4. R Dall, S Longobardi, C Ehrnborg, N Keay, R Napoli, T Rosén, R Baxter, JO Jorgensen, M Kenward, H Orskov, PH Sonksen on behalf of the GH study group, IGF related markers of supraphysiological exogenous GH exposure in 102 healthy young adults.

5. Boroujerdi MA, Dall R, Keay N, Longobardi S, Rosén T, Christiansen JS, Sacca L, Bengtsson BA, Sönksen PH, on behalf of the GH 2000 study group, Simulation of growth hormone dependent marker markers during and following growth hormone treatment.

The following are papers planned for submission in 1999:

1. Jennifer D Wallace, Ross C Cuneo, Per-Arne Lundberg, Nicola Keay, Rolf Dall, Thord Rosén, Jens Otto Jørgensen, Antonio Cittadini, Luigi Sacca, Jens Sandahl Christiansen, Bengt-Åke Bengtsson, Peter H Sönksen, (provisional author list only). Detection of Growth Hormone (GH) abuse in sport: responses of markers of bone and soft tissue turnover to exercise, GH administration and GH withdrawal in trained adult males, Journal of Clinical Endocrinology and Metabolism, submit Jan-Feb 1999

2. Jennifer D Wallace, Christian Strasburger, Per-Arne Lundberg, Lena Carlsson, Ross C Cuneo, Thord Rosén, Bengt-Åke Bengtsson, Peter H Sönksen (provisional author list only). Responses of non-22 kD Growth Hormone (GH) to acute endurance exercise in trained adult males: comparisons of three differing analytic techniques. Journal of Clinical Endocrinology and Metabolism, submit Mar-April 1999.

3. Jennifer D Wallace, Christian Strasburger, Per-Arne Lundberg, Lena Carlsson, Ross C Cuneo, Thord Rosén, Bengt-Åke Bengtsson, Peter H Sönksen (provisional author list only). Responses of non-22 kD Growth Hormone (GH) to exogenous GH administration and withdrawal in exercising, trained adult males: a test for detecting GH abuse in sport. Journal of Clinical Endocrinology and Metabolism, submit April-May 1999.

4. Jennifer D Wallace, Ross C Cuneo, Hans Ørskov, Jens Otto Jørgensen, Rolf Dall, Nicola Keay, Claire Pentecost, Thord Rosén, Antonio Cittadini, Salvatore Longobardi, Luigi Sacca, Jens Sandahl Christiansen, Bengt-Åke Bengtsson, Peter H Sönksen. The effects of Growth Hormone (GH) administration on intermediary metabolism and exercise physiology in trained adult males: does GH enhance exercise efficiency? American Journal of Physiology. Submit June-July 1999.

5. Jennifer Wallace, Ross Cuneo, Martial Saugy, Laurent Rivier, Thord Rosen, Bengt-Ake Bengtsson, Peter Sönksen Urinary GH (? IGF and IGFBPs) responses to rest, acute exercise, recombinant 22 kD GH administration and GH withdrawal in adult males: a potential marker of GH abuse in athletes? Journal: JCEM or GH and IGF Research; submission depends on further assay? Submit late 1999

6. Jennifer Wallace, Ross Cuneo, Steve Bloom, Peter Sönksen. Serum leptin responses to rest, exercise, GH administration and GH withdrawal in adult males. Journal: GH and IGF Research. Submit mid-late 1999.

1. Major Co-operative Links

This study has resulted in the bringing together of most of the European expertise in GH physiology and co-operation with others world-wide as can be seen from the participants detailed above. It has brought together endocrinologists with statisticians, sports medicine & athletes and produced enormous amounts of new and very important physiological information. It has led to the development of a new statistical technique (Professor Philip Brown). It has also enhanced the relationship between academia, IOC, sports authorities and industry to good effect. These links will continue to be forged and will benefit future projects.

7. Contact Details of Each Participant

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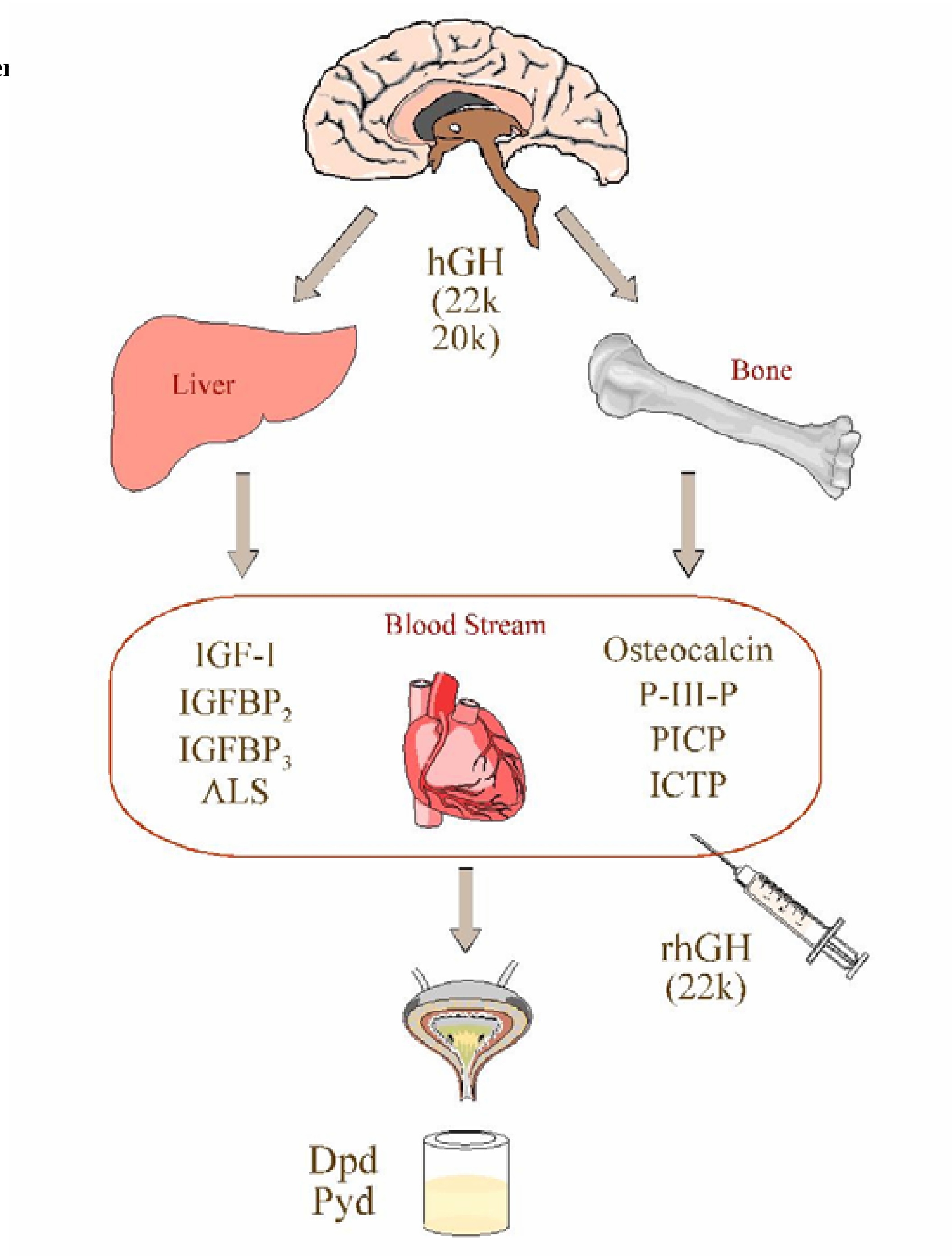
Appendices:

Appendix I: Insulin / IGF-I Axis

Appendix II. JCI Paper

The GH / IGF-I / GH-dependent bone marker axis. Pituitary GH is secreted in pulses mainly in response to exercise, sleep and stress. GH acts on the liver and on bone stimulating the formation of a number of substances ('markers') which are then secreted into the blood stream producing a characteristic response. Exogenous rhGH administration (or something stimulating endogenous GH release) but nothing else, produces a characteristic pattern of response in these markers. This is the basis of the test for GH abuse presented in this report (NB. To be replaced with diagram showing bone as well as liver responses)



Appendix III. Direct Detection of Recombinant Human Growth Hormone (rhGH) Use for Doping in Sports by Differential Immunoassays

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This project is supported by the Bundesinstitut für Sportwissenschaft (BISp), Cologne, Germany (WF 0408/08/02/97)

Background:

Human growth hormone is physiologically produced in the pituitary and released into the bloodstream. Although the 22kDa isoform of hGH represents the major isoform of the pituitary-derived hGH, growth hormone exists in the pituitary and in circulation in multiple isoforms. The second most abundant isoform is the 20kDa variant of hGH, which lacks amino acids 32 to 46 of the 191 amino acids which constitute the 22kDa form of hGH. Acylated, as well as deamidated isoforms and a variable amount of fragments also circulate in blood. The monomeric 22kDa isoform represents roughly 50% of hGH in circulation; all isoforms also appear as dimers and oligomers.

In contrast, recombinant hGH consists only of monomeric and -to a very minor degree- of dimeric and oligomeric 22kDa hGH, which is composed of 191 amino acids. This structural difference between physiological, pituitary-derived growth hormone (representing multiple isoforms), and the homogenous rhGH (composed of only the 22kDa isoform containing 191 amino acids) is exploited for the direct detection of recombinant hGH in blood.

Since 1988, the investigators have raised more than 50 different specific anti-hGH monoclonal antibodies (MABs) of high affinity by immunization with either pituitary-derived or recombinant hGH. This panel of MABs, which are in the sole possession of the investigators, has been used to select antibodies which fulfill one of the following criteria:

1) they are specific for 22kDa hGH, and exhibit either no or only insignificant cross-reactions with other isoforms of hGH, or

2) they represent a "permissive" antibody, binding to all of the different isoforms of hGH derived from the pituitary.

Analytical Technique:

Out of the panel of the previously produced high-affinity anti-hGH antibodies, MAB 5D7 was identified to show a high binding preference for 22kDa hGH over any other isoform of hGH. On the other hand, MAB 1H6 was identified to permissively bind pituitary-derived growth hormone isoforms and have equal reactivity wit 22 kDa- and 20 kDa hGH. Either of these antibodies can be used in a two-site immunoassays.

For this purpose, the antibodies are immobilized to the surface of polystyrene microtiterplates. Known concentrations of growth hormone (calibrator) or unknown serum samples are allowed to incubate with the respective antibodies for 2 hours at ambient temperature; the reaction is then terminated by washing the microtiterplates.

A third anti-hGH MAB, clone 10A7, was identified to specifically bind an epitope distant from the binding regions of both MABs 5D7 and 1H6. This MAB 10A7 is labelled with biotin, and as a second incubation step, is allowed to bind to growth hormone molecules precipitated by the immobilized antibodies.

The signal is detected after a brief additional incubation step with Europium-labeled streptavadin; time-resolved fluorometry is used for quantification. The biotin-streptavidin system serves as a signal amplification step to improve the lower detection limit.

Assay Performance:

Intra-assay coefficient of variation is below 5%; between-assay variation in these immunoassays is below 8%

The lower detection limit of the 22kDa-specific sandwich assay is 0.02 ng/ml, the lower detection limit of the permissive assay for the pituitary isoforms is 0.1 ng/ml.

rhGH Detection:
The ratio of the results measured by the 22kDa-specific assay to those from the permissive assay typically yields between 0.4 and 0.75. In serum samples containing recombinant growth hormone, the ratio typically exceeds 1.0. The fact that the results of 22kDa hGH measurement apparently exceed total GH levels is a consequence of the higher absolute affinity of mAB 5D7 than mAB 1H6. Thus, in a limited incubation time, this antibody binds a higher proportion of molecules from the serum sample. The calculation of the ratio permits judgment of the rhGH content in a given sample. The serum volume required for both assays in duplicate, including eventual confirmatory tests is below 1 ml of serum.

Blinded Confirmatory Experiment:
40 serum samples were kindly provided by Dr. Rolf Dall, Aarhus, within the framework of a collaboration with GH-2000. Half of these 40 sera were derived from pharmaco-dynamic profiles of growth hormone deficient patients receiving an injection of rhGH, while the other half was derived from pituitary stimulation tests. The sera were selected to have comparable absolute hGH concentration ranges, and were provided to the laboratory identified solely by code numbers.
Utilizing the differential immunoassay approach in random order, 20 out of the 40 sera were identified to have a ratio below 0.8, while the other 20 sera had ratios clearly exceeding 1.0. The judgments derived from this analytical approach were reported to GH-2000 and confirmed to be 100% correct.

Results from the "Washout" Study:
Again, sera were provided in a blinded manner and subjected to the above-mentioned growth hormone analysis by differential assays. During the exercise test, after treatment with either placebo or growth hormone (i.e. 3 to 5 hours after the last GH injection), 7 out of 8 volunteers receiving rhGH were detected, whereas the last subject showed borderline ratios, and was thus judged uncertain with respect to rhGH application. Those volunteers who received placebo during the treatment phase were all identified correctly. In essence, one false negative result was reported to the GH-2000; all other judgements were correct. In 2 volunteers who received rhGH, the ratio was still above 1.0 one day later, i.e. 27 - 29 hours after the last GH injection.

Reproducibility and Specificity of a Differential Assay Approach:
The calculated ratio derived from both the permissive and the 22kDa-specific assay was investigated with respect to reproducibility. Within-assay reproducibility of two native pituitary-derived growth hormone-containing serum pools, and two other pools containing a mixture of pituitary- and recombinant-derived hGH was between 3.2 and 5.8% (n=20 per pool). The between-assay variation of 6 runs on 6 different days was between 3.4 and 6.6%.
Insofar as both assays use a sandwich of growth hormone-specific antibodies, and since binding of both antibodies through an antigen is a pre-requisite for signal generation in a sandwich-type two-site immunoassay, the employed immunoassays very specifically detect growth hormone, but no other related proteins or potentially interfering substances.

Discussion:
The above-described differential immunoassay approach to the detection of recombinant hGH in serum samples represents a very promising direct approach to the specific and unequivocal detection of recombinant hGH. The approach makes use of the structural differences between a mixture of isoforms as physiologically secreted by the pituitary gland on the one hand, and the homogenous 22kDa isoform composition of recombinant hGH on the other hand. The approach has proven specific and sensitive in blinded analysis of samples provided by Dr. Rolf Dall, Aarhus, Denmark, and by GH-2000. In the washout study, one false negative judgment was reported to GH-2000: the sera of the volunteer in question yielded borderline ratios between the two assays.

Any direct detection of growth hormone in serum will be restricted to rather short-term detectability as a consequence of the very short half-life of hGH in circulation. After 16 minutes, the concentration of hGH is usually reduced by 50%. Thus, the clearance rate of hGH from circulation is significantly faster than resorption from subcutaneous injection depots. Peak levels of hGH are achieved 2 - 7 hours after subcutaneous hGH injection, with continued resorption from the subcutaneous injection depot thereafter.

Concurrently, after repetitive injections, rhGH suppresses pituitary production of all GH isoforms. Thus, we recently developed a confirmatory test independent from the above described immunoassays. This confirmatory test is based on the specific and direct measurement of 20kDa hGH, the second most abundant isoform of hGH produced by the pituitary gland. In subjecting selected samples from the washout study to this approach, it was demonstrated that injection of rhGH suppresses 20kDa hGH. In the future, this approach could possibly be used as a confirmatory test for suspicious serum samples.

Taken together, the dogma of undetectability of recombinant growth hormone in serum samples no longer holds true. In contrast, rhGH serum samples can be specifically and reliably detected by utilizing the differential immunoassay approach.