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Detection of human growth hormone doping in urine: out of competition tests are necessary[1]

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Abstract

The misuse of human growth hormone (hGH) in sport is deemed to be unethical and dangerous because of various adverse effects. Thus, it has been added to the International Olympic Committee list of banned substances. Until now, the very low concentration of hGH in the urine made its measurement difficult using classical methodology. Indeed, for routine diagnosis, only plasma measurements were available. However, unlike blood samples, urine is generally provided in abundant quantities and is, at present, the only body fluid allowed to be analysed in sport doping controls. A recently developed enzyme-linked immunosorbent assay (Norditest) makes it now possible, without any extraction, to measure urinary hGH (u-hGH) in a dynamic range of 2–50 ng hGH/l. In our protocol, untreated and treated non-athlete volunteers were followed. Some of them received therapeutical doses of recombinant hGH (Norditropin) for one week either intramuscularly (three increasing doses) or subcutaneously (12 I.U. every day). The u-hGH excretion after treatment showed dramatic increases of 50–100 times the basal values and returned to almost the mean normal level after 24 h. u-hGH was also measured in samples provided by the anti-doping controls at major and minor competitions. Depending on the type of efforts made during the competition, the hGH concentration in urine was dramatically increased. Insulin-like growth factor binding proteins and β2-microglobulins in urine and/or in blood could be necessary for the correct investigation of any hGH doping test procedure.

Keywords: Human growth hormone

1. Introduction

Human growth hormone (hGH) is produced naturally by the pituitary gland. In humans hGH is heterogeneous, the major component being a single-chain peptide of 191 amino acids stabilised by two disulphide bonds, with a molecular mass of approximately 22 000 (22K).

Recombinant hGH (r-hGH), produced by recombinant DNA technology, has an amino acid sequence identical to the pituitary derived one.

Beside the classical drugs (stimulants, analgesic, etc.), the main international sport federations and the International Olympic Committee (IOC) have instituted comprehensive control procedures which are directed mainly towards the eradication of anabolic steroid use. As the efficiency of detection of steroids

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[1] Dedicated to the memory of the late Dr. Donike.

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increases, some athletes stopped their consumption in favour of other anabolic agents. Non-official reports indicate that hGH is the current choice for many athletes. So far, there is no reliable nor scientific evidence of the use of hGH by athletes, but only indirect or anecdotal indications. This is the case in American football, track and field and body-building. hGH is placed on the IOC list of banned substances in sports.

There are two main reasons to believe that the use of hGH in sports could soon become widespread:

(a) the promotion of biotechnology products has drastically increased the safe supplies and availability of r-hGH; (b) recent reports suggest an increase in non-muscular fat-free mass [1,2] and thus an increased resistance against tendon rupture in young athletes treated with supra physiological doses of hGH [3].

At present, urine remains the only biological sample available for doping tests. Therefore the anti-doping laboratories should control hGH in urine.

It has recently been shown that new progress in enzyme-linked immunosorbent assay (ELISA) technology made the detection of hGH in urine reliable, even though its concentration is a 1000-fold lower than in blood. These measurements have been shown to be very useful for the investigations of abnormalities of GH excretion [4,5]. However, urinary-hGH (u-hGH) excretion depends not only on plasma GH concentrations, but also on the renal handling of the hormone [6]. The kidney plays a major role in the removal of the circulating low molecular mass proteins and peptides [7], including peptide hormones such as GH. The mean hGH production in normal subjects ranges from 500 to 875 μg/day and about 0.01–0.001% of that amount is excreted in the urine. This is due to extensive proximal renal tubular reabsorption and catabolism within the tubular epithelial cells [8]. The tubular uptake process of GH seems to be an energy-dependent adsorptive endocytic process and is characterized by a high capacity as compared with the normal filtered load of small proteins. Impairment of tubular uptake of proteins can give rise to massive peptiduria, up to 10 000-fold greater than in normal conditions. In a diabetic population, the excretion of hGH was found to be 100–1000-fold higher than in the normal population. This huge increase in u-hGH could not be attributed to the 2–3-fold elevation in plasma concentration, but rather to a defect of the renal handling of GH in diabetic patients [6]. The same authors found a strong positive correlation between u-hGH and urinary β2-microglobulin in diabetic cases indicating that both factors are handled by the kidney in the same manner.

Proteinuria due to strenuous exercise is a commonly observed phenomenon in humans. It seems to be related to the intensity of exercise rather than to its duration. This excretion of proteins in urine is transient with a half-time of approximately 1 h [9]. It is dependant on the type and the size of proteins. A correlation can be drawn between proteinuria and the intensity of exercise expressed by the lactate formed [10]. The increased clearance of plasma proteins and peptides during strenuous effort suggests an increase in glomerular permeability and a partial inhibition of tubular reabsorption of these molecules [9].

1.1. Aim of the study

The aim of the study was first to measure hGH concentration in urine provided by volunteers before and after r-hGH treatment, and then, to analyse urine provided to us by the Swiss anti-doping control system (at the national and international level) and by volunteers after sport practice. We would also carry out measurements on urine collected at controls during the Olympic games in 1992. They were provided to us by the Barcelona laboratory. On all of these competition samples, the effect of exercise on the urinary hGH concentration was evaluated qualitatively and quantitatively.

1.2. u-hGH measurements

Any test used to measure hGH in urine should be very sensitive and specific since the concentration of the hormone is usually very low in this biological fluid. For a mean integrated plasma concentration of 3.5 ng/ml, 400 μg GH would be filtered per 24 h [11]. However, only a small proportion will finally appear in the urine since most of it is absorbed and metabolised in the renal tubules. The urinary fraction

of GH is around 0.001–0.01% of the circulating amount.

2. Experimental

2.1. Volunteers in competition

Before and after a classic 17 km race, 130 male volunteers produced urine in order to measure the influence of effort on their urinary hormonal profile. Beside urine collection, several clinical observations were collected which will not be reported here.

2.2. Anonymous samples from the anti-doping control system

u-hGH was measured in urine provided by anonymous athletes practising different sports in which competitions were organised at international level. The main Olympic sports are represented in this study. Professor J. Segura, Head of the Barcelona anti-doping laboratory provided us with 100 competition samples from the Olympic Games of Barcelona (1992). These samples were originally controlled and found to be free of doping agents, and they were chosen to represent different sport disciplines and different types of efforts.

2.3. Protocol of hGH application

Eighteen healthy male volunteers (age: 18–50 yr; weight: 65–80 kg; non-athletes) were observed and their urine collected during the three days before the treatment. They were then treated in the morning with r-hGH (Norditropin) following the protocol defined below:

- First treatment[2]: 5 (6 in one case) subcutaneous injections of 12 international units (I.U.) hGH (one every day)
- Second treatment[2]: 3 intra-muscular injections (every two days) with increasing dosage, (6, 12 and 24 I.U. respectively.)

Aliquots of all the urines collected separately were stored at 4°C until analysed. The analyses were performed within 2 weeks after collection.

[2] The ethical commission of the medical Faculty of our University approved this protocol.

2.4. Assays

The ELISA NordiTest™ u-hGH (Fig. 1) incorporates two antibodies: one polyclonal guinea pig antibody labelled with alkaline phosphatase, and one monoclonal mouse antibody adsorbed to the solid-phase in the microtest plate format and an enzyme amplification stage (AMPAK [12]. The latter considerably increased the sensitivity of the assay compared to other types of tests. The method is applicable to direct assay of hGH in urine (i.e. unconcentrated, undialysed urine) and detects mainly 22K-hGH, the most abundant excreted form. Its high specificity ensures that the naturally occurring forms of hGH are determined as well as the commercially available hGHs (r-hGH and methionine-hGH), but not the structurally related hormones [13]. Urinary insulin-like growth factor binding protein-3 (IGFBP-3) radio-immunoassays were graciously obtained from Nichols Institute Diagnostics (Geneva, Switzerland), and β2-microglobulin was measured by enzyme immunoassay (Cobas Core, Roche Diagnostics, Basel, Switzerland).

2.5. High-performance capillary electrophoresis (HPCE)

The experiments were performed with a Hewlett–Packard (Walbronn, Germany) HP^{3D}CE system. This system consists of a capillary electrophoresis unit equipped with a diode-array detector, an autosampler and a high-velocity air-cooled capillary cartridge. The HP^{3D}CE Chemstation software was used for instrument control, data acquisition and data analysis. The HP capillary was of fused-silica with a 560 mm length to detector (645 mm total length) with 50 μm I.D.

The sample (11.8 nl) was injected by pressure ($5 \cdot 10^{-2}$ MPa·s) and electrophoresis was performed at constant voltage of 44.6 V·mm^{-1}. All runs were

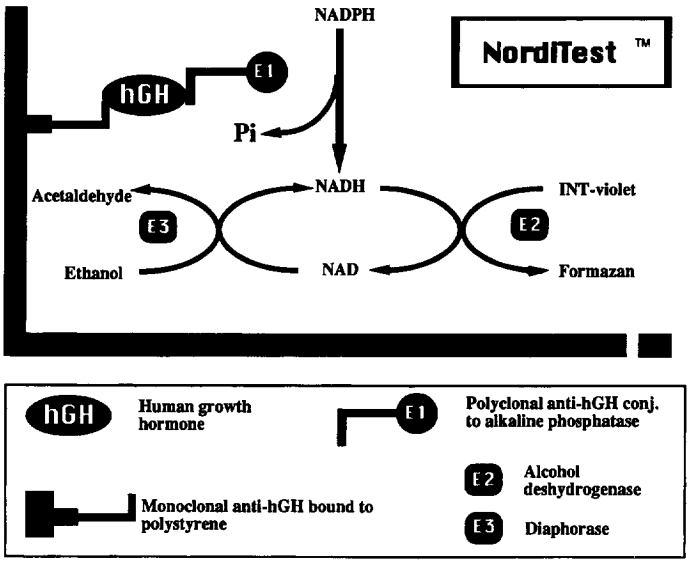

Fig. 1. Principle of urine assay procedure with Norditest. The color development is stopped by addition of acid and measured at 490 nm with a reference wavelength of 650 nm.

carried out at 23°C with a 100 m*M* phosphate buffer (NaH_2PO_4–Na_2HPO_4, pH 8.0). Detection was performed using a diode-array detector; the electrophoreograms were monitored at 200 nm (10 nm bandwidth) and a reference signal at 350 nm (100 nm band width).

3. Results and discussion

3.1. u-hGH concentration of the control group

The frequency distribution of the u-hGH concentrations (Fig. 2, 196 subjects, 499 determinations)

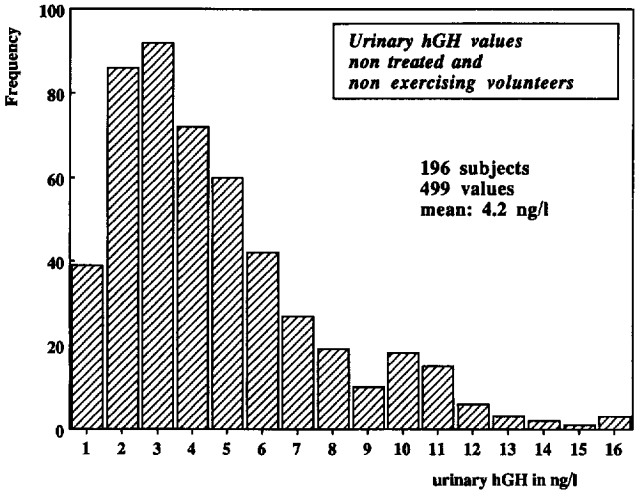

Fig. 2. Frequency distribution of u-hGH values (499) measured in 196 healthy subjects.

shows that most values are lower than 16 ng/l, the mean value being 4.2 ng/l (S.D.=3.2 ng/l). However, the variability in u-hGH concentration is quite important. Some of the subjects exhibit changes from 1 to 20 ng/l and this variability is generally not dependent on the specific gravity of urine or creatinine content [14]. Except for the effect of exercise (see below), there was no correlation between the urinary hGH concentration and specific events of normal life (sleep, meal, psychologic stress, etc.) as observed in blood [11].

3.2. Non-athlete volunteers treated with Norditropin

Two types of treatment were applied to the volunteers. Usually, for therapeutical purposes, hGH is applied subcutaneously six days a week for a long period of time. In the present case, beside subcutaneous. applications, intramuscular injections were also applied in order to mimic what we believe to be a common habit in different risk populations (especially body-builders).

3.3. Subcutaneous treatment

Following subcutaneous injections, all subjects present the same pattern of hGH profile, whatever the parameter used for expression of the hGH excretion (direct concentration, total excretion, corrected by the creatinine content; data not shown).

Fig. 3 shows a typical excretion profile for one volunteer who has received a daily 12 I.U. subcutaneous treatment for six days. It appears that 6 to 12 h after each subcutaneous application, u-hGH rises to a value at least 50- to 100-fold higher than the normal level. However, already after 24 h, the concentration returns back to its initial level.

Table 1 shows the mean values of the peak urine concentrations for nine volunteers receiving subcutaneous injections over a five-day period. There is no significant difference between the days of treatment and it is obvious that the variability between individuals is quite important. The daily maximum peaks of hGH concentration reached by seven volunteers during the five-day treatment clearly shows that after the same program of injections, the excretion pattern is quite variable (Fig. 4).

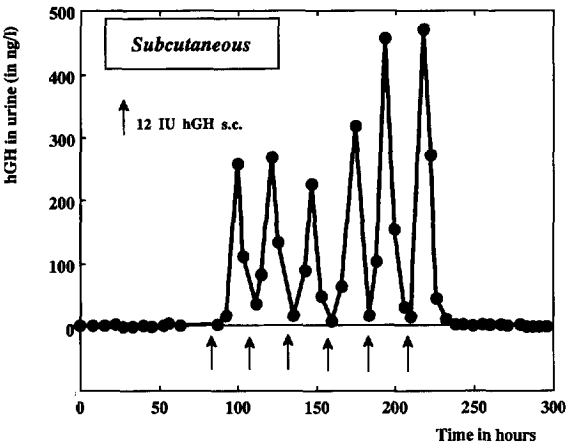

Fig. 3. u-hGH concentration (in ng/l) before and during a subcutaneous treatment. The volunteer received every day for 6 days 12 I.U. r-hGH subcutaneously in the morning. The arrows show when the hormone was injected during the experiment.

Table 1
Mean values (n=9) of the peak concentration after 12 I.U. r-hGH subcutaneous injections

Injections	Mean hGH in urine (±S.D.)
Before treatment	4.3 ± 4.1
1st day	136 ± 84
2nd day	185 ± 83
3rd day	147 ± 67
4th day	182 ± 73
5th day	202 ±121

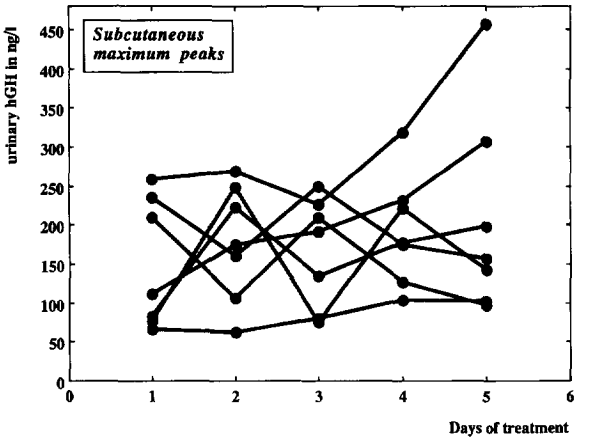

Fig. 4. u-hGH peak concentration curves of 7 volunteers during a treatment of 5 daily subcutaneous injections (12 I.U. r-hGH each). On the graph, only the maxima concentrations reached every day by the volunteers are represented.

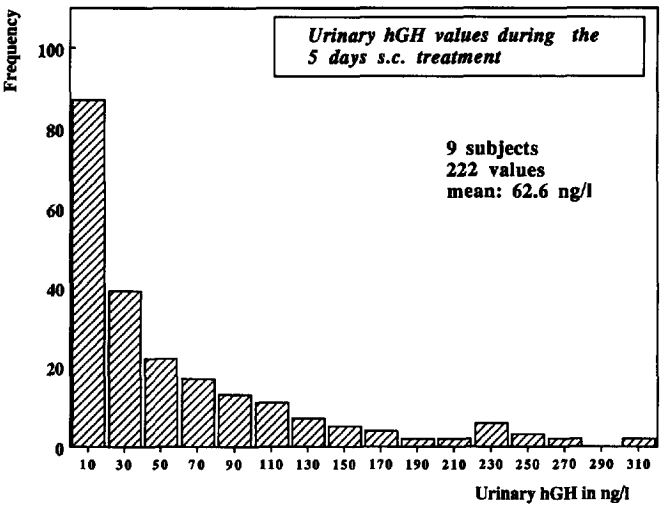

Fig. 5. Frequency distribution of u-hGH values (n=222) measured in all 9 treated subjects spot urines during the 5-day subcutaneous treatment (12 I.U. r-hGH per injection).

Fig. 5 shows the frequency distribution of urine concentrations during the subcutaneous treatment. This distribution is composed of high values measured in urine collected during the first hour after the injections, and normal values found in urine collected when the concentration had returned to the baseline level (more than 12 h after the injections). Although the type of distribution of both control and treated populations does not allow a correct statistical analysis, it can be calculated that 33% of the values obtained during the treatment are lower than the highest control value (1.6 ng/l). This means that in 64% of the cases, the u-hGH concentration can be considered as suspicious. The determination of any cut-off level in a screening procedure for hGH should take into account this parameter.

3.4. Intramuscular treatment

The intramuscular injections were repeated three times within a 48-h period interval. From 6 I.U. the first day, the dosage was increased to 12, then 24 I.U. in the following days of treatment, in order to test the dose response effect (Fig. 6).

For most subjects, the values of the maximum concentration increase along with the dosage. As observed in the subcutaneous treatment, the peak values appears almost 6–12 h after each injection and disappears completely after 24 h. Here also, the inter-variability is high (Fig. 7). Two volunteers did not even show any increase in the urine concentration between the 12 I.U. and the 24 I.U. applications.

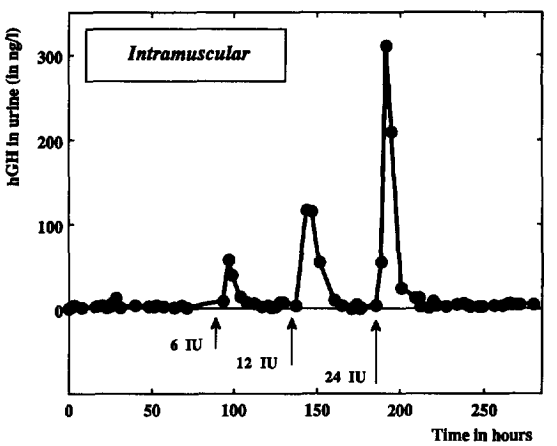

Fig. 6. u-hGH concentration (ng/l) before and during an intramuscular treatment. The volunteer was treated in the morning every two days. He received 3 increasing doses of r-hGH intramuscularly, respectively 6, 12 or 24 I.U. by day. The arrows show when the hormone was applied during the experiment.

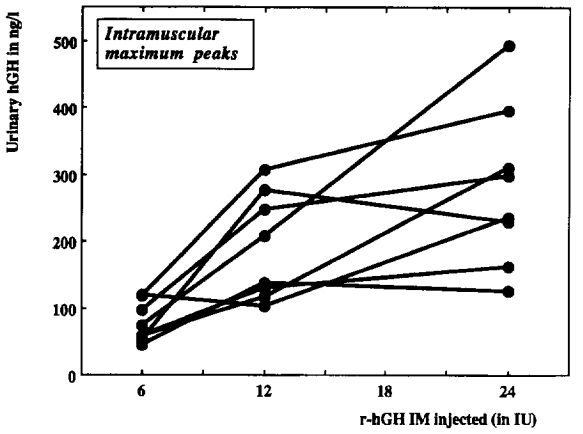

Fig. 7. u-hGH peak concentrations curves of eight volunteers after receiving increasing dosages of r-hGH (6, 12 and 24 I.U.) by intramuscular injections. The time interval between the injections is 48 h. On the graph, only the maxima concentrations reached each day of treatment by the volunteers are represented.

3.5. Urinary hGH and competition

3.5.1. Anti-doping anonymous samples

u-hGH was measured in anonymous spot urine collected after several competitions held in Switzerland at an international level. Results are summarised in Fig. 8. In competitions of track and field and rowing, the mean concentration of the hormone is very high, and differs considerably from other sports

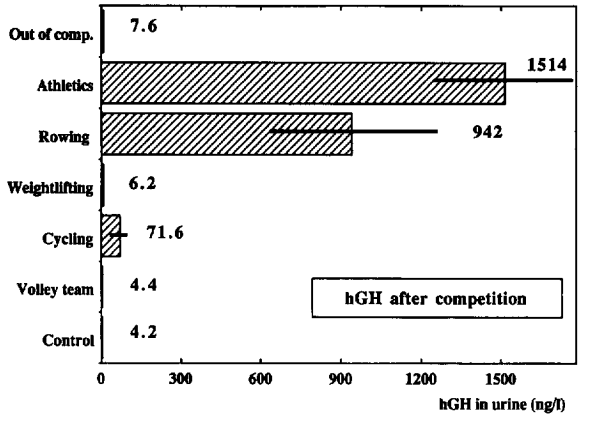

Fig. 8. Mean values (±S.D.) of u-hGH measured in anonymous urines collected after major competitions in different sports. Number of samples per groups were: 50 for out of competition; 60 for athletics; 30 for rowing; 30 for weightlifting; 60 for cycling; 179 for volley team; 499 for controls.

and from the controls. Looking closely into the different disciplines in track and field, it seems that middle distance runners are providing the highest values. This type of exercise can be compared to rowing: a relatively short (lasting between 1 and 15 min long) but very intense effort.

The specificity of the test was checked at that time because of the very high response obtained in these particular disciplines. Biochemical investigations did not reveal any unspecific reactions. Moreover, the samples from major "Grand Prix" track and field events were measured in parallel in Lausanne and Oslo using two independent commercial immunoassays systems, based on two different antibodies and detection reaction (Norditest from Novo Nordisk and Delfia from Pharmacia). The comparison between these two assays gave similar results.

Because the Olympic games generally provide a large number of samples from different types of sports during a short period of time and under the same conditions of collection, 130 samples (13 disciplines with 10 samples each) from Barcelona 1992 were analysed to verify this kind of exercise-dependent increase in u-hGH. Data shown in Fig. 9 also demonstrate that the short and violent type of effort performed by athletes tends to increase hGH excretion.

3.6. Urinary hGH before and after a 17-km race

In order to evaluate this effect on a controlled population, 130 athletes (a mixed population of national and regional level of runners) were tested for u-hGH before and after a 17-km race. To account for the differences in hydration status of each individual, the results are normalised with the creatinine content. The results show (Fig. 10) that after the race, most of the athletes exhibit an elevated concentration of u-hGH. The mean values measured before and after the race are also significantly different: before the race: 4.0±0.2 ng/l (±S.E.), 12.6±1.3 ng/g creatinine; after the race: 62.8±7.9 ng/l (±S.E.), 65.2±10.4 ng/g creatinine. If the mean value (4.0 ng/l) obtained before the race is similar to the one measured in the control group (Fig. 2), the concentrations of hGH reached by some of the runners after competition are close to those

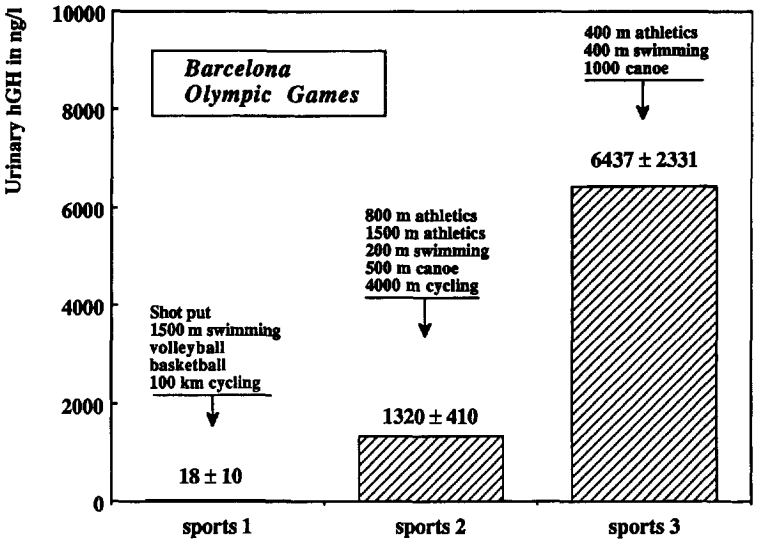

Fig. 9. Mean values (±S.D.) of u-hGH measured in urines collected during the Barcelona Olympic games. Total number of samples: 130.

measured in the treated volunteers. This observed high excretion could not simply be explained by an increase in circulating hGH in the body. Under these conditions, the increase in hGH excretion in urine is surely increased due to modification of the renal function during that kind of effort.

It was demonstrated that both glomerular filtration and tubular reabsorption are implicated in a higher clearance of proteins by strenuous exercises [9]. Proteinuria is induced and is more dependent on the intensity of the effort than its duration. The size of the protein is also a determinant: while the clearance of albumin (M_r~69 000) increases nearly 30-fold under the influence of strenuous exertion, lysozyme (M_r~15 000) and β2-microglobulin (M_r~11 500) increases 160-fold compared to resting values. It can be assumed that hGH (M_r~22 000) is affected in a similar way according to its size. Thus, the kidneys play a major role in the removal of the circulating low-molecular-mass proteins and peptides [7] including peptide hormones. Turner et al. [6] already found such huge increases of u-hGH in diabetic patients presenting a defect in the renal handling of hGH.

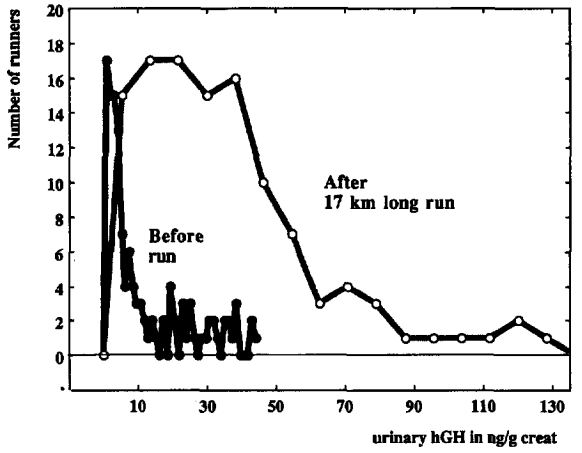

Fig. 10. Frequency distributions of u-hGH (in ng/g creatinine) in a population of 130 athletes before and after a competition. In the after competition values, ten high values (between 130 and 1000 ng/g creatinine) are not shown to make the figure easier to read.

Those authors suggest further that β2-microglobulin and hGH excretions are very similar, because of the strong correlation between these two parameters obtained in diabetes mellitus cases.

This hypothesis was also tested on the samples from the Olympic games (Fig. 11). Three groups of urine with respectively low, medium and high hGH values were studied as to their β2-microglobulin content in urine: there is a clear relation between these two parameters. As normal values of urinary β2-microglobulin for healthy man are lower than 200 μg/l [15], our data clearly indicate that the

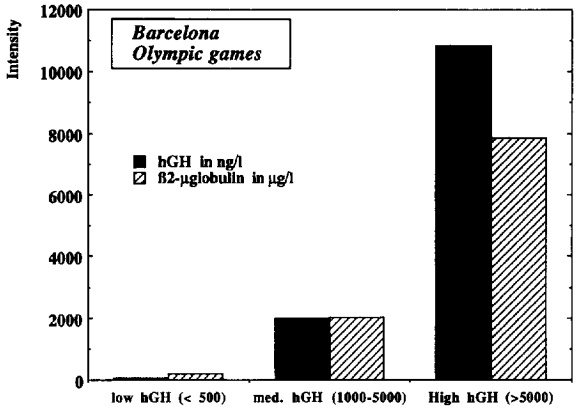

Fig. 11. Mean values of u-hGH and β2-microglobulin measured in urines collected during the Barcelona Olympic games (total $n=60$).

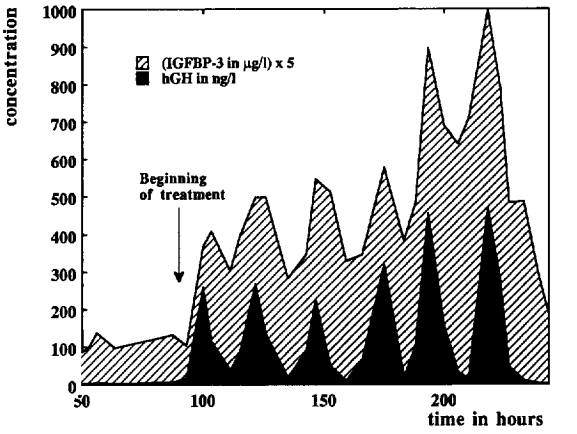

Fig. 12. hGH and IGFBP-3 concentrations in urine from a r-hGH subcutaneously treated volunteer (6 daily subcutaneous injections of 12 I.U. r-hGH each).

excretion of this peptide is dependent on physical exercise. Furthermore, urine β2-microglobulin measurements could be used as an indicator of kidney function.

4. Perspectives for the future

4.1. IGFBP-3 and other binding proteins

Recent development of specific radio immunoassays for IGFBP-3 have permitted its accurate quantification in several biological fluids [16]. The measurement of this binding protein, seems to be now more useful clinically than the value of the insulin-like growth factor-1 (IGF-1), which is produced under the control of hGH. The knowledge of both hGH and IGFBP-3 levels in serum has already been used for diagnosis of GH deficiency [16].

We have measured this binding protein in urine samples from one hGH treated volunteer (one subcutaneous injection of 12 I.U. r-hGH every day for six days) (Fig. 12). IGFBP-3 increases during the treatment. The discrimination between the normal level and what is obtained after the treatment is smaller than the change in hGH values. However, the absolute concentration of IGFBP-3 in urine seems to increase slightly with time. Further experiments are now under investigation in our laboratory to test the effect of a long term treatment of r-hGH on IGFBP-3 values.

The same samples were tested for β2-microglobulin concentrations (not shown on the graph) which remained normal (<200 μg/l) for all the samples during the treatment.

4.2. Blood and CE

The extremely low concentration of hGH in urine will force the analyst to investigate other parameters in urine and other biological samples. The hGH concentration in blood is 1000-fold higher than that in urine. In normal situations, hGH is present in the body under several molecular forms. The ratio between these forms could be monitored to detect doping. The biosynthetic form is a pure 22K as in the natural one, whereas in the blood, another molecular form (20K) is also present. Our hypothesis is that after injection of the biosynthetic hormone, the ratio between the two forms 22K/20K will change in blood in favour of the 22K. The measurement of the two forms could be best performed with CE as shown by Arcelloni et al. [17]. The electropherogram (UV detection) shown in Fig. 13 was obtained with a concentration of 22K r-hGH higher than 1000 times the natural blood concentration. Clearly, the sensitivity of detection of that methodology has to be improved.

Fig. 13. r-hGH electropherogram. Technical conditions: buffer, NaH_2PO_4–Na_2HPO_4 100 m*M* (pH 8.0); capillary, 560 mm/645 mm, I.D. 50 μm; voltage and pressure, 25 kV, 23°C, 5×10^{-2} MPa·s (11.8 nl); sample, r-hGH in water (440 μg/ml); instrument, Hewlett–Packard 3D CE.

5. Conclusion

5.1. Sensitivity and specificity

The new ELISA test used in the present study is well suited to the measurements of u-hGH in control and athlete subjects. The main advantage of this kit is its high sensitivity. Another advantage is the possibility of u-hGH measurements without any preparation of the sample (no concentration, no dialysis). Several tests of specificity (data not shown here) demonstrated that this kit was not cross-reacting with similar types of hormones and that no other urine compounds were affecting the detection system.

5.2. u-hGH after application

After both intramuscular and subcutaneous injections, the urinary hGH concentration increases significantly by a factor depending on the dosage. In both cases, the baseline value is recovered within less than 24 h. It can be concluded from this experiment that any doping with hGH in that range of dosage could be detected only for a short period of time and if the athlete was not competing before the urine collection. It is then necessary to investigate further parameters involved in that process. The measurements of IGF-1 and bone markers, far example, in those urines are now in process.

5.3. Is u-hGH screening usable for the detection of hGH doping?

Of course, the answer is not yet definitive. To complete our present study, several experiments are still under investigation. IGF-1, IGFBPs and other parameters implicated in growth processes are now being measured on the same urine samples.

However, it can be postulated that u-hGH measurements in competition tests are not valuable for the detection of GH doping in cases of strenuous effort. However, another urine collection in the morning before the competition or out of competition could be taken, the expected values could then be considered to be in a normal range of hGH concentration. In case of doping however, if the injection was performed the day before the urine collection (less than 12 to 18 h), an elevated concentration of growth hormone should be detected in that urine.

5.4. Proposal for hGH doping detection

Based on the results presented in this paper, some important parameters should be taken into account in order to propose a screening procedure for hGH doping detection:

(1) Collected urine should be out of competition samples.

(2) β2-Microglobulin should be analysed to attest that no alteration of the kidney function occurred (due to strenuous effort or any kind of kidney dysfunction before the sample was taken).

(3) u-hGH should be analysed: if the kidney function is normal and the concentration is higher than 20 ng/l (cut-off based on Norditest, this has certainly to be established and could be different for another EIA), further investigations could take place immediately: (a) follow up of the athlete with several blood and urine samplings; (b) analysis of as many secondary parameters as possible like: (i) binding proteins and IGF-1 in urine and blood, (ii) bone metabolism markers.

To attest the efficiency of such a strategy, development of specific assays will be necessary and further experiments are in progress with real cases in body building, a sport believed to be especially exposed to the extensive use of hGH.

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