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Detection of human growth hormone in urine of athletes

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Introduction :

Human growth hormone (HGH, somatotropin) is a polypeptide hormone released by the hypophysis which controls growth and effects the protein-, lipid- and carbohydrate metabolism. HGH stimulates the protein biosynthesis which makes it a potential anabolic doping agent. In 1989 the medical commission of the IOC put HGH on the list of banned substances.

Li and co-workers isolated and characterised HGH first in 1956[1]. The hypophysis, which contains high concentrations of HGH (5-15 mg/g), releases two types of HGH, a 22 kDa type, consisting of 191 amino acids with two disulphide-bonds between the positions 53 and 165 and between 182 and 189, and a 20 kDa type (176 AA; AAs 32-46 are missing) with the same growth promoting effect as the 22 kDa HGH but decreased metabolic impact.

HGH tends to form dimeres (MW 45 kDa) as well as tri-, tetra- and pentameres (MW in excess of 60 kDa)[2].

HGH is rapidly metabolised in the liver with a half-life of ca. 20 min. Small amounts of HGH, up to 10 pg/ml of urine, are cleared by the kidney. This clearance is strongly influenced by physical strain.

Until the mid 80's HGH was extracted from human hypophyses. Nowadays recombinant HGH is expressed by prokaryotes like E. coli and its increased availability made it easier for athletes to abuse HGH for the following reasons:

- increase of raw muscle mass and power
- preservation of muscle mass
- increased effect of anabolic steroids
- influence of growth of young athletes[3].

This paper tries to explain whether serum and urinary concentrations of HGH[4] can be compared, how physical strain in training and competition influences the concentrations of HGH and whether it makes sense to incorporate the determination of HGH concentrations into the regular doping analysis.

Experimental:

Standards and chemicals:

Recombinant human growth hormone (rHGH) was purchased from KABI VITRUM PHARMACIA (Sweden), the PD-10 gel filtration columns from PHARMACIA (Sweden), Bovine serum albumin (BSA) from SIGMA (Germany) and the chemiluminescence immunometric assays for HGH in serum and urine from NICHOLS INSTITUTE DIAGNOSTICS (Germany). All other chemicals used were of analytical grade.

Sampling procedures:

Urine samples were collected in PE-containers, containing 1.2 ml of 20% BSA in 0.01 mol/l PBS/0.1% NaN_3), from 5 male athletes (running (2), weight lift. (1), judo (1), football (1)) before and after physical strain. Furthermore blood and corresponding urine samples were collected from one volunteer before and after a single dose of rHGH (4 I.U.; s.c.). The urine samples were collected as before, the blood sample were collected in serum collection tubes (CORVAC), centrifuged and the serum withdrawn and deep frozen at -20 °C. Also all of the in-competition test samples of the 1993 IAAF World Championships in Stuttgart were used for this work.

Extraction of HGH from Urine:

PD-10 columns were equilibrated with 25 ml 0.01 mol/l PBS/0.1 NaN_3. The urine sample was centrifuged for 15 min. at ca. 1200x g and 1 ml of the supernatant was applied to the column. Then 1.5 ml 0.01 mol/l PBS/0.1 NaN_3 were applied. The first 2.5 ml eluate were discarded. The HGH is eluted with another 2.5 ml 0.01 mol/l PBS/0.1 NaN_3.

Chemiluminescence immunometric assay:

Urinary HGH: 1 ml each of the urine samples (treated as above) or of the urinary HGH kit standard solutions (treated like the urines) were pipetted in duplicate into 5 ml borosilicon test tubes (12x75 mm; SARSTEDT, Germany). Then polystyrene balls, coated with a polyclonal HGH antibody, were added to each tube and the tubes were

incubated at room temp. on a rotator at ca. 180 rpm for 18 h. After the first incubation the tubes were washed and aspirated with 4x2 ml of dest. water.

For the second incubation 200 µl of a solution of a monoclonal mouse antibody to HGH with a chemiluminescence marker (acridinium-ester) were added to each tube which then were incubated at room temp. on a rotator at 180 rpm for 6 h. After this second incubation the tubes were washed and aspirated successively with 0.5 ml PBS and 4x2 ml dest. water.

Serum HGH: 50 µl each of the serum samples or of the serum HGH kit standard solutions were pipetted in duplicate into 5 ml borosilicon test tubes (12x75 mm; SARSTEDT, Germany). Then 200 µl of a solution of a monoclonal mouse antibody to HGH with a chemiluminescence marker (acridinium-ester) and a polystyrene ball, coated with a polyclonal HGH antibody, were added to each tube which then were incubated at room temp. on a rotator at ca. 180 rpm for 1 h. After this the tubes were washed and aspirated successively with 0.5 ml PBS and 4x2 ml dest. water.

Then concentrations were determined right away with a BERTHOLD luminometer.

Results:

Influence of physical strain on HGH concentrations in urine:

Urine samples of five male athletes were collected just before, right after a workout and some time later. The intensity of the workout wasn't determined. Table 1 shows the results of this experiment:

It can be seen that the urinary HGH concentrations after physical strain were higher than before.

Athlete	workout type	Conc. [pg/ml]
MK.1	before	5
MK.2	weightlifting	26
MK.3	1.5h after	122
AB.1	before	4
AB.2	10K run	168
AB.3	4h after	14
CK.1	before	7
CK.2	halfmarathon	181
CK.3	4.5h after	22
TZ.1	before	9
TZ.2	judo	70
TZ.3	1.5h after	29
KG.1	before	0
KG.2	football	38
KG.3	3.5h after	11

Table 1: uHGH conc. before and after physical strain

For comparison, urinary HGH concentrations of 16 male volunteers (athletes and non-athletes; ages: 25 to 42 yrs.; rested) showed levels between 0 and 12 pg/ml with a mean of 6.1±3.9 pg/ml.

Influence of physical strain on HGH concentrations in blood:

The influence of physical strain on HGH concentrations in blood is determined by the following experiment. One of the athletes (MK) of the previous experiment underwent three different treadmill-tests on three consecutive days.
In the first test the athlete started with a velocity of 2.5 m/s. The pace was increased 0.5 m/s every three minutes up to a velocity of 5.0 m/s. Blood was collected just before the test, after every increase of pace and every 10 min. up to one hour after the test.
In the second test the athlete ran for 10 min. at 2.5 m/s then the velocity was increased to 5.5 m/s. The athlete was to maintain this pace for as long as possible, which was 2 min. Blood was collected again just before the warmup, after the warmup, after the 2 min. at 5.5 m/s and every 10 min. up to one hour afterwards.
The third test was an endurance test. The athlete ran for 20 min. at 3.5 m/s and 10 min. at 4.0 m/s. Blood was collected just before the test and then every 10 min. up to one hour afterwards. Table 2 and diagram 1 show the results :

Time	1. test	Time	2. test	Time	3. test
	sHGH		sHGH		sHGH
	[ng/ml]		[ng/ml]		[ng/ml]
0	0.063	0	3.3	0	0.12
3	0.067	10	2.7	10	0.18
6	0.075	12	3.3	20	0.97
9	0.058	22	9.2	30	9.1
12	0.096	32	10.2	40	11
15	0.13	42	12.1	50	10.8
18	0.35	52	10.9	60	10.2
28	3	62	6.5	70	6.8
38	5.2	72	4.4	80	5
48	3.7			90	3.3
58	1.5				
68	0.79				
78	0.47				

Table 2 : Spontaneous sHGH concentrations after physical strain (The grey areas indicate excercise periodes, the white areas rest periodes)

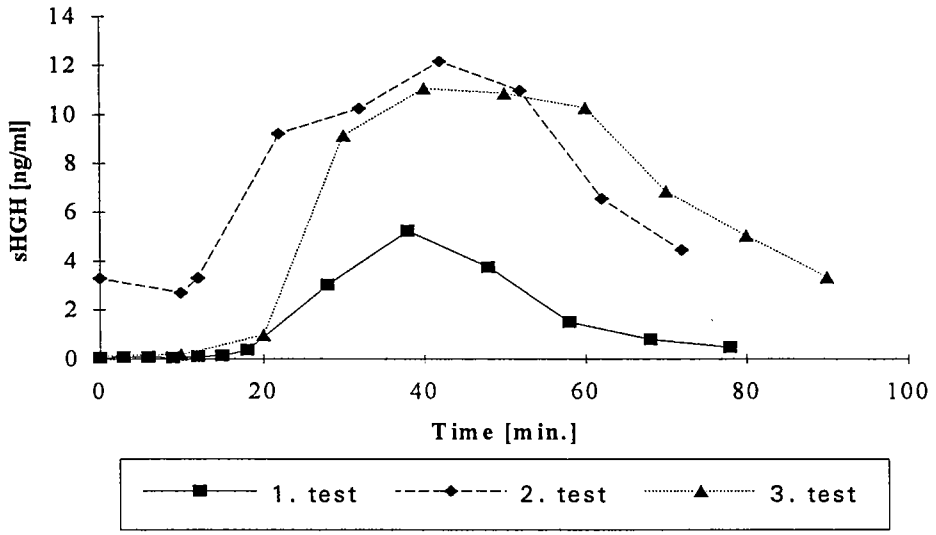

Diagram 1 : Spontaneous sHGH concentrations after physical strain

Excercises with high lactic acid production like the second and third test show higher sHGH concentrations. There seems to be a positive correlation between lactic acid production and endogenous HGH excretion.

Parallelism between serum and urinary concentrations before and after HGH application:

To see whether there is a parallelism between serum and urinary concentrations and how a HGH application influences these, the following experiment has been conducted. Urine and blood samples of athlete of the previous experiment (MK) were collected over a period of 11 h. Table 3 and diagrams 2 and 3 show concentrations and excretion rates:

Time	urinary HGH			serum HGH
	Conc.	Volume	Excretion rate	Conc.
	[pg/ml]	[ml]	[pg/h]	[ng/ml]
08:45	2.9	90	116	0.07
09:45	21.9	50	1093	0.13
10:45	16.5	40	662	0.08
11:45	3.2	385	1217	0.09
13:45	4.1	255	528	0.16
15:45	9.2	160	738	0.09
17:45	6.1	230	702	0.93
19.45	10.3	74	383	0.15

Table 3: Spontaeous urinary and serum HGH concentrations from athlete MK

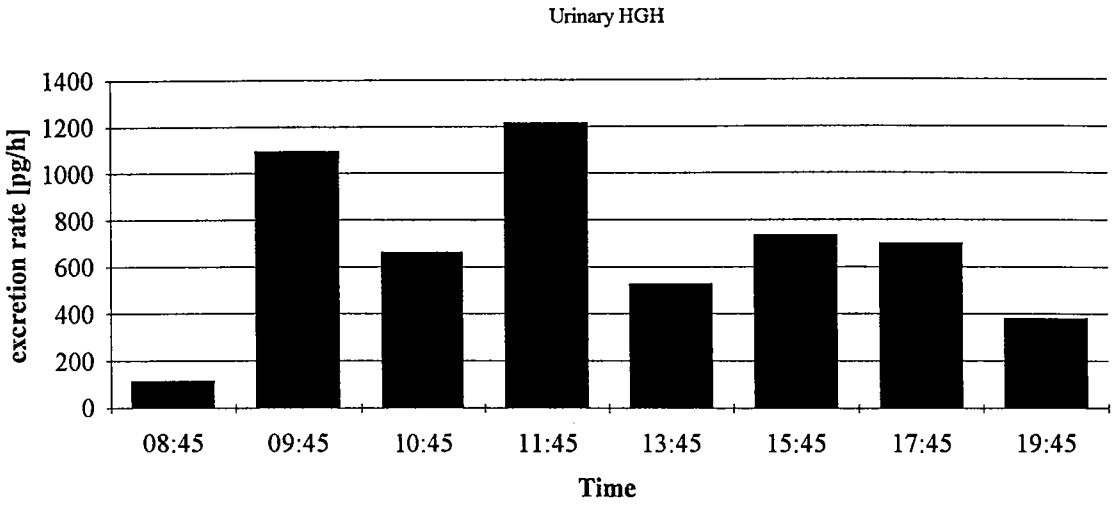

Diagram 2: Spontaneous urinary HGH excretion rates

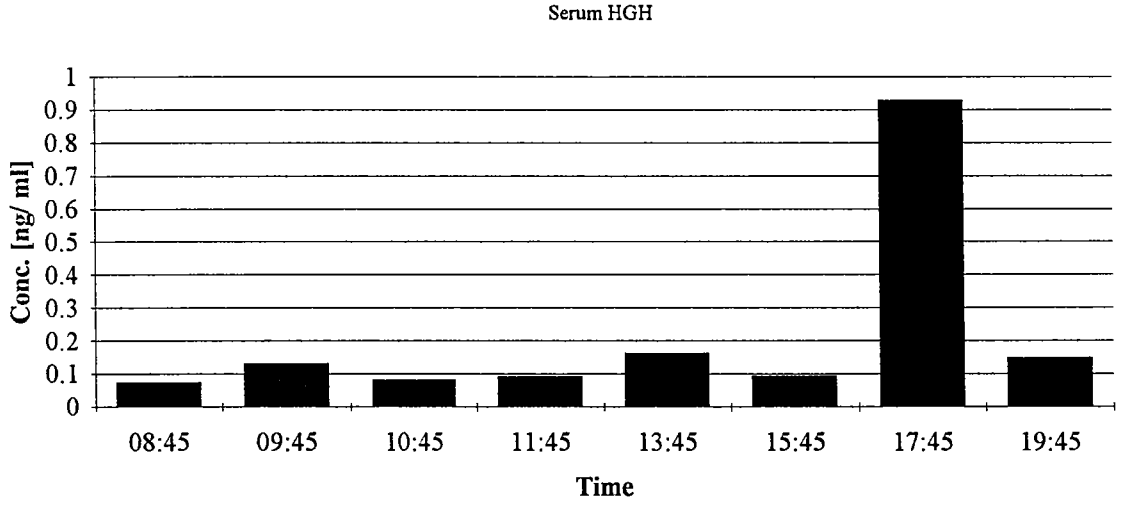

Diagram 3: Spontaneous serum HGH concentrations

The urinary and serum concentration levels show great variation over this 11 h time period.

Next a single dose of 4 I.U. HGH s.c. (09:15 a.m.) was applied to the same person. A blood and a urine sample was collected right before application and at different times afterwards. Tables 4 and 5 and diagram 4 show the results.

Time	Conc.	Volume	Excretion rate
h	pg/ml	ml	pg/h
-2.75	54.0	380	1643
-0.25	1.3	130	66
1	7.9	70	444
3	26	130	1690
5	46.4	120	2784
7	27.6	100	1381
9	10.4	120	627
11	3.1	360	558
13	6.2	100	308
15	2.2	60	67
17	2.7	100	133
19	3.7	110	203
21	0.9	120	52

Table 4 : Urinary HGH excretion rates before and after 4 I..U. HGH

Time	Conc.	Time	Conc.
[h]	[ng/ml]	[h]	[ng/ml]
-0.25	0.17	6	8.70
0	0.29	7	6.05
0.5	5.63	8	3.29
1	9.63	9	2.19
2	17.79	10	1.18
3	21.63	11	0.88
4	17.86	12	0.66
5	11.32	13	0.51
		23.75	0.15

Table 5: Serum HGH concentrations before and after 4 I.U. HGH

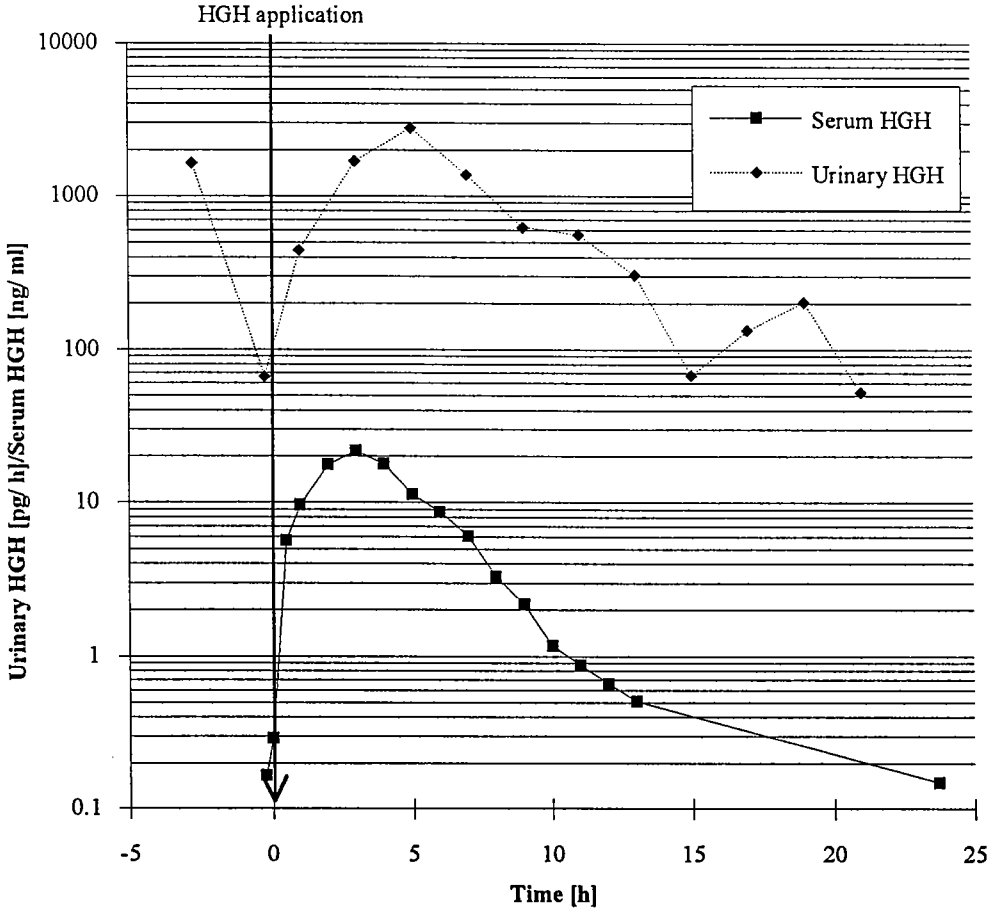

Diagram 4: Urinary and serum HGH concentrations before and after application of 4 I.U. HGH

In serum the highest concentration was detected 3 h after application. 11 h after application the serum concentration had returned back to a normal level. For the urinary concentration the highest excretion rate was detected 5 h after application. All other samples showed excretion rates similar to or much smaller than the morning urine sample.

Urinary HGH concentrations in samples taken during the 1993 IAAF WC Stuttgart:

216 urine samples of the 1993 IAAF WC Stuttgart drawn from athletes of all disciplines were checked for HGH concentrations. Table 6 and diagram 5 show a statistical evaluation of the collected data:

Discipline	Occurrences in urinary HGH concentration range			
	0-50 pg/ml	50-150 pg/ml	150-350 pg/ml	>350 pg/ml
100m	13	4	1	6
4x100m	4	2	0	2
100m Hurdles	1	0	2	1
110m Hurdles	0	1	0	5
200m	3	1	1	7
400m	3	0	0	7
4x400m	1	0	0	17
400m Hurdles	0	0	0	7
1500m	0	0	0	9
3000m	1	0	1	6
5000m	0	0	0	4
10000m	0	4	0	4
Marathon	2	4	1	1
10km Walk	1	0	3	0
20km Walk	3	3	0	0
50km Walk	4	1	0	0
800 (Heptathlon)	0	2	0	11
Decathlon	0	0	0	4
Discus throw	8	0	0	0
High jump	9	0	0	0
Hammer throw	4	0	0	0
Javelin throw	6	1	1	0
Javelin throw	8	0	0	0
Pole vault	4	0	0	0
Shot put	8	0	0	0
Triple jump	8	2	0	0

Table 6: Occurrences for different uHGH conc. ranges divided into disciplines

The most occurrences for very high urinary HGH concentrations were in the middle distance running disciplines. There is also a high lactic acid production in those disciplines. Our own experiments (data not shown) show that there is a positive correlation between endogenous HGH excretion and lactic acid production.

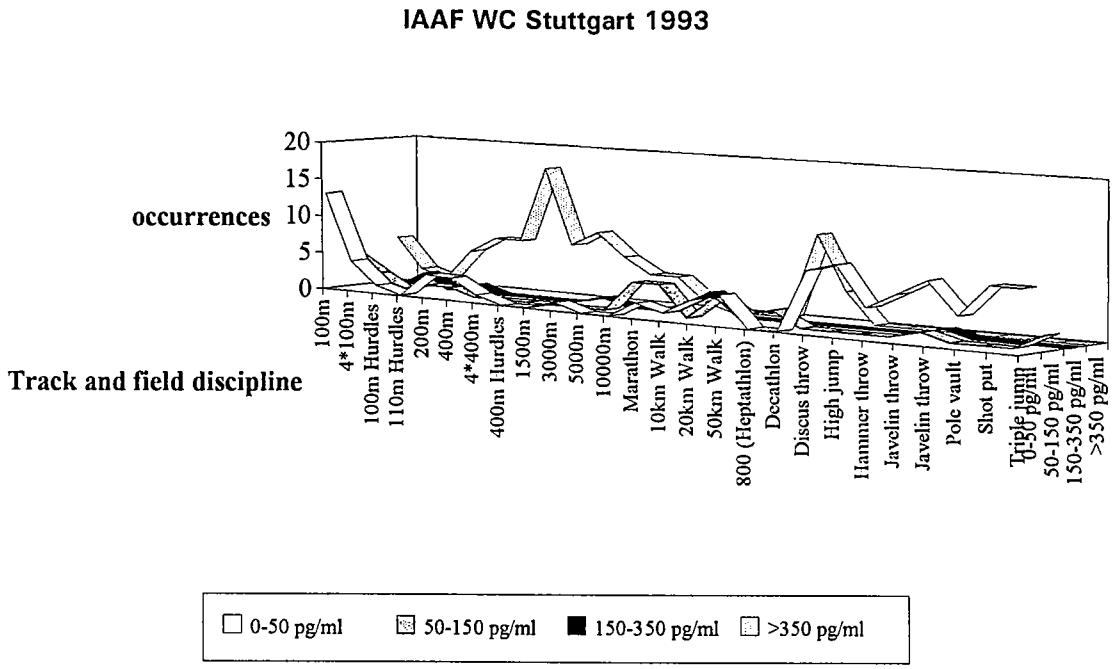

Diagram 5: Occurrences for different uHGH conc. ranges divided into disciplines

Discussion:

Influence of physical strain on HGH concentrations in urine:

The urinary HGH concentrations of 21 rested subjects (16 male volunteers (untrained) and five athletes (table 1)) range from 0 to 12 pg/ml with a mean of 5.8 ± 3.7 pg/ml. The urinary HGH concentrations of 5 athletes, urine samples collected up to 4.5 h after a workout, range from 11 to 181 pg/ml with a mean of 73 ± 67 pg/ml.

Physical strain obviously influences the urinary HGH concentrations and raises them well above the level for rested subjects.

Influence of physical strain on HGH concentrations in blood:

Physical strain strongly influenced the serum HGH concentration of athlete MK. The more exhausting the work load is, the higher the sHGH concentration. The highest sHGH concentration is found in the third treadmill-test as is the highest lactic acid

concentration in blood. So there seem to be a positive correlation between lactic acid production and endogenous HGH excretion.

Parallelism between serum and urinary concentrations before and after HGH application:

The concentrations and excretion rates of HGH in urine and serum show great variations but no parallelism over an 11 h time period without an application of HGH. The maximum of the urinary excretion with 1217 pg/h is 10.5 times higher than the minimum excretion with 116 pg/h. Serum HGH shows a similar behaviour with a maximum of 0.93 pg/ml, which is 12.5 times higher than the minimum of 0.074 pg/ml. But the max. uHGH excretion rate and the max. sHGH concentration lie 6 h apart.

The picture is a little different after an HGH application of 4 I.E. s.c.. Now one can see an increase in concentration and excretion rate almost parallel for urine and serum with maxima (uHGH 2784 pg/h at 5 h; sHGH 21.6 ng/ml at 3 h) that are about two hours apart. But already after 11 h the concentrations reach normal levels, so that the detection of HGH application by concentration in routine urine samples becomes impossible.

Urinary HGH concentrations in samples taken during the 1993 IAAF WC Stuttgart:

91 of the 216 in-competition samples from the 1993 IAAF WC Stuttgart show uHGH concentrations higher than 350 pg/ml. 61 of these 91 come from the running distances from 400 to 5000 m. Only one sample of the throw and jump disciplines falls within this range.

Incorporation of the determination of uHGH concentrations into the regular doping analysis :

"Doping" is defined as use of substances listed in the " List of banned substances" by the IOC. HGH or Somatotropin is listed there since 1989. So far it is impossible to detect the abuse of HGH, since rHGH and endogenous HGH cannot be differentiated because of identical amino acid sequences. For now the only way to detect HGH application would be to establish reference ranges for the concentrations in blood or urine. In out of competition control the collection of blood samples is up to now not allowed. That leaves the doping control laboratories with urine as a matrix.

This work shows that the concentration and excretion rate of uHGH varies widely even without application of HGH. Establishing a reference range or a maximum concentration, above which a sample would be called "positive", is not possible for uHGH.

This is not true for serum HGH. The highest concentration of sHGH after physical strain was still smaller than the highest concentration of sHGH after application of a single dose of 4 I.U.. Because of the lower variation, blood reference ranges might be established if a large enough population can be tested.

Acknowledgements:

Special thanks to my dear colleague Andreas Breidbach for helping me writing this paper.

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[1]Li C H et al; Human growth hormon; Molecular and cell biochem.; 46(1982); 31-41

[2]Harper, Martin, Mayes, Rodwell; Medizinische Biochemie; Springer Verlag; Berlin/Heidelberg/New York; 1987; 601-613

[3]Kley H K; Leistungsbeeinflußung durch Peptidehormone: Wachstumshormon, ein anaboles Dopingmittel?; lecture held at the 2nd meet of the workgroup"Dopingfragen" on Sept. 12th 1984; Federal institute of sport science

[4]Abbreviations used: uHGH - urinaty Human Growth Hormone: sHGH - serum Human Growth Hormone