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# Enrichment and immunoprecipitation of 22 kDa human growth hormone spiked into human urine

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**Approaches to detect whether an athlete has used growth hormone have been intensely investigated by sport organizations for 20 years. This effort has led to a human growth hormone (hGH) isoform ratio test in serum that has been approved by WADA and deployed at three Olympic Games, although a positive case has yet to be reported. We set out to determine whether the ratio test could be applied to urine. First we investigated various ways to extract hGH from spiked urine. We were able to recover 95% using selective centrifugal concentration. This fraction was then subjected to four different commercially available immunoprecipitation kits. The highest yield was obtained with the Invitrogen Dynabeads Protein G kit. Nevertheless it is apparent that these methods do not recover enough hGH for subsequent analysis by mass spectrometry. With further effort greater recovery of the 22 kDa isoform might be achieved, however it is very unlikely that the 20 kDa isoform could be detected. This method may be significantly improved by the application of both nanoparticle and aptamer technology.** 

**Keywords:** doping in sport; human growth hormone; biological markers; isoforms; immunoprecipitation

## Introduction

Media reports, police investigations and confessions of former athletes regularly show that human growth hormone (hGH) is widely used to enhance athletic performance.[1,2] Athletes focus on the reports that hGH increases lean body mass, burns body fat and shortens recovery times between training sessions. Several scientific studies have been completed and there remains disagreement as to whether hGH use is effective.[1] Clearly, though, GH-deficient patients gain strength and muscle mass when hGH is replaced.[3,4] In addition there is evidence that hGH in combination with anabolic androgenic steroids (AAS), or when administered to former users of AAS, does enhance performance.[5]

Twenty years ago the International Olympic Committee (IOC) added hGH to the list of banned substances and in 1992 Professor Sonksen, an expert on hGH, joined the IOC Medical Commission to work on GH issues. In 1996 a grant was awarded by the IOC and the EU to Sonksen to discover GH-dependent markers.[6] When the World Anti-doping Agency (WADA) was formed by the IOC in 1999, hGH detection continued to be a priority. The years of intense effort on the GH detection project indicate both the high degree of difficulty and the determination of the sports community to find a solution.[6] Progress has been slow but steady.

The two most evolved methods are (1) a measurement of the ratio of the 22 kDa isoform of hGH to total hGH in serum and (2) the quantitation of two GH dependent markers (PIIIP and IGF-I). Both methods are based on detection of relevant substances in serum. In the first method the administration of the recombinant 22 kDa isoform of hGH results in high levels in serum, and by feedback inhibition the levels of pituitary isoforms decline, thus altering the 22 kDa/total hGH ratio.[7,8] This ratio method is WADA-approved and was used at the Athens Olympic Games in 2004, as well as all subsequent Olympic Games; however, there are no official or unofficial reports of positive cases.[6] This is generally attributed to the short duration of the effect of hGH administration on the ratio.

The second method measures the concentrations of biomarkers (PIIIP and IGFI) in serum by immunoassay in response to hGH administration.[9–13] This method is well validated and supported in the literature. It awaits WADA approval. Note that neither of these two methods are based on identification of hGH by an unambiguous analytical method. The hGH isoform ratio test is carried out in serum with validated immunoassays. In contrast there are no validated assays for hGH isoforms in urine largely due to the low levels of hGH in urine. The ready availability of urine in large volume makes it the body fluid of choice for doping control.[14] Urine-based testing for peptide or glycopeptide hormone doping agents such as insulin,[15] EPO,[16,17] and GnRH[18] have been successfully validated. Saugy *et al*.[19] described the detection of hGH in urine by immunoassay in 2007 although it has not been validated or approved by WADA.

We began work on hGH more than two years ago when two professional sport leagues asked if it might be possible to develop a urine test. After considerable discussion on the high degree of difficulty and low likelihood of success, we began to investigate the feasibility of detecting hGH in human urine by mass spectrometry. The project has two phases: the first is the optimization of a process to enrich and concentrate hGH in urine and the second is to identify hGH from a variety of endogenous and recombinant sources using an Orbitrap mass spectrometer, an extremely sensitive type of

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LC/MS. This report focuses on immunoprecipitation methods[20,21] for extracting and concentrating hGH that has been spiked into human urine.

## Materials and Methods

### Purified proteins

The native 22 kDa isoform of hGH was obtained from the National Hormone and Peptide Program, Torrance, California. Monoclonal antibodies to hGH used for western blotting and immunoprecipitation included mAb 1067, R&D Systems, Minneapolis, MN and mAb A36020 BiosPacific, Emeryville, CA. Polyclonal antibodies used for western blotting and immuno-precipitation included AF1067 Goat from R&D, AFP-C11981A Rabbit from National Hormone and Peptide Program, and sc-10 365 Goat from Santa Cruz Biotechnology, Santa Cruz, CA. Secondary antibodies for western blotting were purchased from Invitrogen, Carlsbad, CA: HRP-Rabbit Anti-Goat (#81-1620), HRP-Goat Anti-Mouse (#81-6520), HRP-Goat Anti-Rabbit (#65-6120).

### Urine enrichment/concentration by centrifugal membrane separation

In order to concentrate hGH in urine, 20 mL aliquots were spiked with increasing amounts of native 22 kDa hGH (50 to 1000 ng), then added sequentially to centrifugal membrane filters (Sartorius Biotech, Goettingen, Germany) with 10 and 50 kDa molecular weight cutoffs (MWCO). In Figure 1, the retentate from the 10 kDa MWCO filter was recovered, diluted in 20 mL of immuno-precipitation buffer (0.01 M PBS, pH 7.2, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$), and applied to the 50 kDa MWCO filter. All centrifugations were carried out for 30 minutes at 5000 g at room temperature. The amount of native 22 kDa spiked into 20 mL urine samples was gradually reduced over the course of the work, to 100 ng total in the final Invitrogen Protein G Dynabeads experiment described below. This was the smallest amount of 22 kDa that could be detected in 20 mL of urine by all four kits. The concentrations of hGH were determined by Immulite 1000 (Siemens Healthcare Diagnostics, Deerfield, IL).

### Visualization

*Electrophoresis*

All gels/blots were run on the XCell SureLock Mini gel/blotting system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, in 4–12% Bis-Tris gels with 2-(N-morpholino)ethanesulfonic acid (MES) as the running buffer. Gels were stained by Coomassie blue stain.

*Western blotting*

Proteins were electrotransferred from the gels to PVDF membranes (Immobilon-P, Millipore, Bedford, MA). Immunological detection was performed in all cases with anti-hGH polyclonal antibodies (Rabbit AFP-C11981A, National Hormone and Peptide Program, Torrance, CA) at 1 : 25 000 dilution overnight at 4 °C with rotation. After extensive washing with Tris-buffered saline/Tween-20, the blots were further incubated for 60 minutes at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Upon completion, a chemiluminescent substrate (ChemiGlow, Alpha Innotech, San Leandro, CA) was added and the image acquired by a FluorChem Imager (Alpha Innnotech, San Leandro, CA).

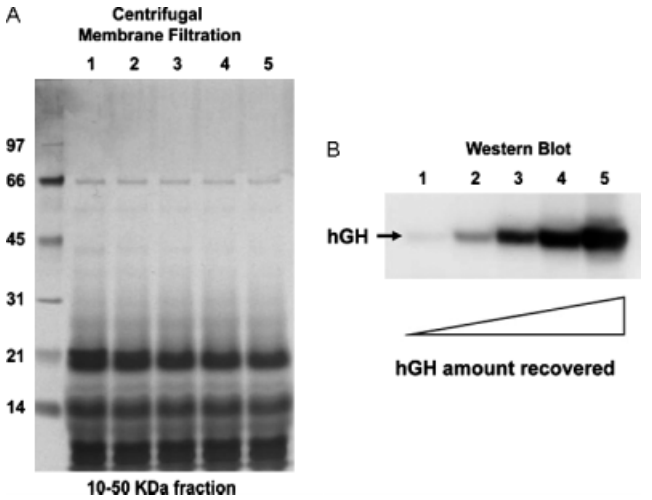


**Figure 1.** SDS-page gel stained with Coomassie blue and western blot analysis of urine samples spiked with various amounts of 22 kDa hGH. The SDS-page gel (Figure 1A, lanes 1–5) show the results for five 20 mL urine samples spiked with 50, 100, 250, 500 and 1000 ng, respectively and processed sequentially with 10 kDa and 50 kDa MWCO centrifugal membrane filters. The final total volume is approximately 100 µL. In Figure 1B a western blot was performed on the five samples. It shows that as the amount of hGH increases the amount of it that can be visualized also increases. For lane 1 the hGH is barely visible indicating the threshold for combined recovery and detection is approximately 50 ng/20 mL or 2.5 ng/mL.

*Densitometry*

Densitometry was performed on the acquired image as per the manufacturer's suggestions, with the Alpha Innotech AlphaEaseFC software. Briefly, the gel image was divided into nine lanes of equal width. Areas of peak density (bands) were automatically located and quantified using the 'Autogrid' option on the software. Net area counts were obtained by using the 'Auto Base' background subtraction option. Data from each lane were exported onto a spreadsheet to facilitate calculations. Percentage rates of recovery for each procedure were calculated by comparing the hGH signal in each lane to that found for a standard curve of 5, 25, 50 and 100 ng native 22 kDa hGH.

### Immunoprecipitation methods

The Classic IP Protein G Kit was obtained from Pierce Biotech, Rockford, IL. Cat. No. 45 218. All of Pierce's buffers were used according to the kit instructions. Briefly, 10.0 µg of an hGH-specific antibody was incubated with 100 µL of enriched urine sample to form a complex at 4 °C for 2 h with rotation. The complex was captured by adding the sample to a cup containing 0.4 mL Protein G resin and incubated for 2 h at 4 °C. The captured sample was washed several times and eluted at pH 3.0.

The Direct IP Protein G Kit was obtained from Pierce Biotech, Rockford, IL. Cat. No. 26 148. The buffers were used according to kit instructions. Briefly, 20 µL of AminoLink Plus Coupling resin, 10 µg of an hGH-specific antibody and 6.0 mL of crosslinking agent (sodium cyanoborohydride) are added to a column and incubated at room temperature for 2 h with rotation. Following several washes, 100 mL of enriched urine sample were added to the spin column and incubated for 2 h at 4 °C. The captured sample was washed several times and eluted at pH 3.0.

The Dynabeads M-280 Tosylactivated kit was obtained from Invitrogen Corp., Carlsbad, CA, Cat. No.142.04. All of Invitrogen's

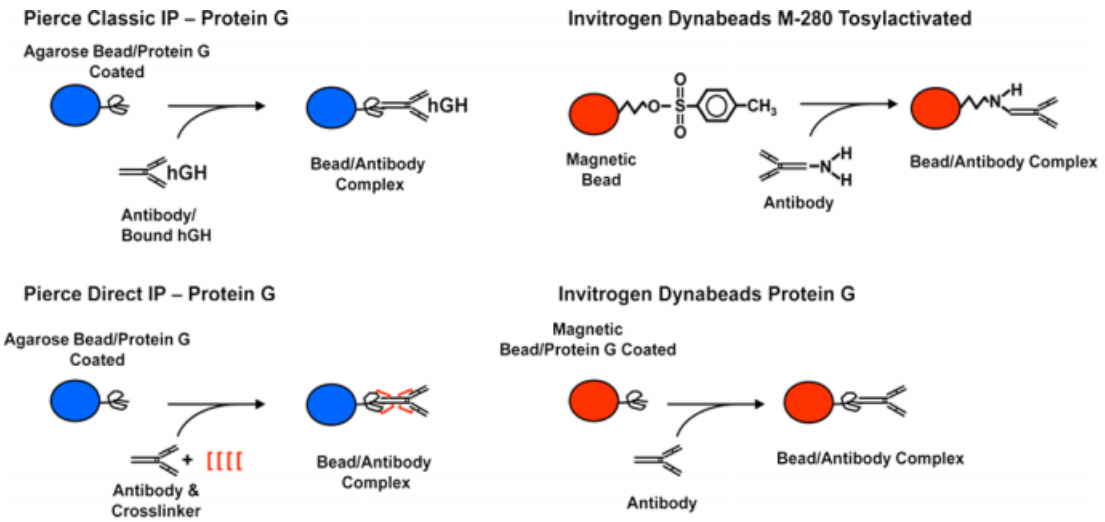


**Figure 2.** Diagrams explaining the differences between the four kits used to extract hGH from urine. In Figure 2A (upper left) the enriched and concentrated sample is incubated with anti-hGH antibodies. Then agarose beads coated with protein G are added. This results in a bead/antibody complex. After washing the hGH is eluted at pH = 3. In Figure 2B (lower left) the process is the same except that a crosslinking reagent and the antibodies were incubated with the agarose beads coated with protein G. In Figure 2C tosyl activated magnetic beads are incubated first with anti-hGH antibody, then added to the enriched and concentrated sample containing hGH. A magnet is used to hold the magnetized bead/anti-body complex while the complex is washed. The hGH is eluted from the bead/antibody complex at pH = 3. In Figure 2D magnetized beads coated with protein G are incubated with anti-hGH antibody. After this complex is washed, it is added to the enriched and concentrated sample. After washing the hGH is eluted at pH = 3.

buffers were used per kit instructions unless otherwise noted. Briefly, 10 µg of an hGH-specific antibody were added to 1 mg of Dynabeads in coupling buffer and incubated at room temperature for 48 h with rotation. Following several washes the Dynabeads/antibody complex was added to a 100 µL enriched urine sample and incubated for 2 h at 4 °C. The sample was eluted in 0.1 M citric acid at pH 3.0.

The Immunoprecipitation Kit – Protein G Dynabeads was obtained from Invitrogen Corp., Carlsbad, CA. Cat. No.100.07D. All Invitrogen buffers were used per kit instructions. Briefly, 1.5 mg of Protein G Dynabeads were incubated with 10 µg of an hGH-specific antibody and incubated for 10 min at room temperature with rotation. Following several washes the bound bead/antibody complex was added to 100 µL of enriched urine sample, mixed by pipetting, and incubated for 2 h at 4 °C with rotation. The captured bead/Protein G/antigen complex was washed several times and eluted at pH 3.0 with rotation at room temperature.

## Results

In this series of experiments the strategy was: first, the enrichment and concentration of hGH in a 20.0 mL urine sample, second, recovery of the hGH by immunoprecipitation and third, the detection of hGH by western blot.

### Enrichment/concentration of the hGH fraction of urine proteins

Figure 1 shows the fractionation of urinary proteins by centrifugal membrane separation with selective molecular weight cutoffs of 10 and 50 kDa. In this way hGH is enriched in a fraction of proteins larger than 10 and smaller than 50 kDa. The complexity of the samples was assessed by SDS-PAGE (Figure 1A) and the enrichment of hGH by western blot (Figure 1B). The 50 kDa filter was chosen as the upper limit for two reasons; first, to remove as much as possible of the larger protein species, i.e., serum albumin and immunoglobulins, and second, to include a 44 kDa dimer of the 22 kDa isoform of hGH frequently observed at pH 7.2 in our experiments. The figure indicates that the protein content of the urine has been simplified and that hGH is enriched in proportion to the amount added. As a control for sample loss, we measured the concentration of hGH by Immulite 1000, which has an effective concentration range of 50 pg/mL to 40 ng/mL, before and after the concentration step and found >95% recovery for the process.

### Recovery of hGH from an enriched sample

In order to ascertain the best method to recover hGH from a spiked and concentrated urine sample we compared four widely used and commercially available immunoprecipitation methods. All follow the same basic format. First, the antibodies are crosslinked to a solid phase. Next, the unbound antibody is washed off and finally the bead/antibody complex is used to immunoprecipitate the antigen from a sample. The final complex is washed several times to remove nonspecific material and the antigen is eluted, usually at low pH in a glycine buffer.

Initial method comparisons included two monoclonal and three polyclonal antibodies to hGH (see 'Materials and methods'). Where possible method components were normalized, i.e., manufacturer's suggestions as to solid phase necessary to bind 10 µg of antibody, reaction temperatures, and 100 ng hGH spike. We evaluated and optimized our results based upon the following criteria: percentage of the antibody crosslinked to solid phase; percentage of the hGH present in the enriched urine sample that was captured by the antibody and, finally, whether the antigen could be efficiently eluted from the antibody without significant interference from a co-eluting antibody. We monitored the crosslinking step by SDS-PAGE gels and Coomassie staining. Human growth hormone capture was analysed by western blot and densitometry.

Table 1 summarizes the overall performance of the kits. Crosslinking of the antibody was monitored by Coomassie

**Table 1.** Comparison of four methods for immunoprecipitating hGH

| Criteria | Pierce classic IP Protein G | Pierce Direct IP Protein G | Invitrogen Dynabeads M-280 | Invitrogen Dynabeads Protein G |
|---|---|---|---|---|
| Crosslink efficiency | <15 | 40 | <15 | 85+ |
| Elution | poor | fair | poor | good |
| Antigen capture (% recovery) | <5 | <5 | <25 | 50+ |
| Co-elution of antibody | poor | good | poor | good |

gel comparing crosslinked bead/antibody with the amount of unbound antibody remaining in the crosslinking buffer post reaction. Western blots were used to monitor the remaining criteria. Efficient elution was measured by comparing the eluate to a concentration curve and any hGH remaining bound on the beads/antigen complex after elution. A poor elution indicates that 50% of the recovered sample remains on the beads. A fair elution indicates that 15–20% of the samples remains and a good elution indicates that elution was nearly quantitative. Antigen capture success was measured by comparing the eluate to a concentration curve of native 22 kDa by western blot.

It was also possible to observe antibody fragments that co-eluted with hGH and were non-specifically bound by the primary antibody in the western blot. Significant antibody signal was rated as poor and little to no signal was judged to be good. This is a major concern as non-crosslinked antibody can result in reduced recovery of hGH and suppression of the hGH signal by mass spectrometry.

The initial crosslink between the antibody and the solid phase is a critical step in this process. Figure 2 shows a simple diagram of this step for each product compared in the study. This process affects the success of the antigen capture step. The antibody must be efficiently and tightly bound to the solid phase and it must not readily wash off. Each kit evaluation was performed with five antibodies, two monoclonals and three polyclonals, based upon the same criteria: crosslinking, recovery and elution. Experiments were carried out simultaneously with additional monoclonal and polyclonal antibodies. For the sake of brevity the process is described in detail only for a single monoclonal antibody, R&D Systems mAb 1067.

First we examined the efficiency of the crosslink between the solid phase of each kit, agarose or magnetic beads, and the antibodies. Figure 3 shows the results of the experiment using the R&D mAb 1067. All of these results are in pairs. The odd-numbered lanes contain the successfully crosslinked solid phase/antibody complex and the even-numbered lanes contain all residual antibody not successfully crosslinked. If the crosslinking was not successful the odd lanes will appear blank. The Pierce Classic IP – Protein G (lanes 1–2) and Invitrogen Dynabeads M-280 (lanes 5–6) gave poor results because of the large amount of antibody remaining in the buffer following the reaction (lanes 2 and 6). The Pierce Direct IP – Protein G kit (lanes 3 and 4) was an improvement in that it incorporates a chemical crosslinking reagent into the reaction that covalently binds the antibody to the Protein G coating the agarose bead. This makes the solid phase/antibody bond resistant to downstream handling and co-elution. Lanes 3 and 4 of Figure 3 indicates that approximately 40% of the antibody is bound to the bead.

Lanes 7–12 of Figure 3 illustrate an efficient crosslinking reaction. Lanes 7, 9 and 11 indicate a crosslink between 1.5 mg of Invitrogen Protein G Dynabeads and 10, 20 and 50 μg mAb 1067

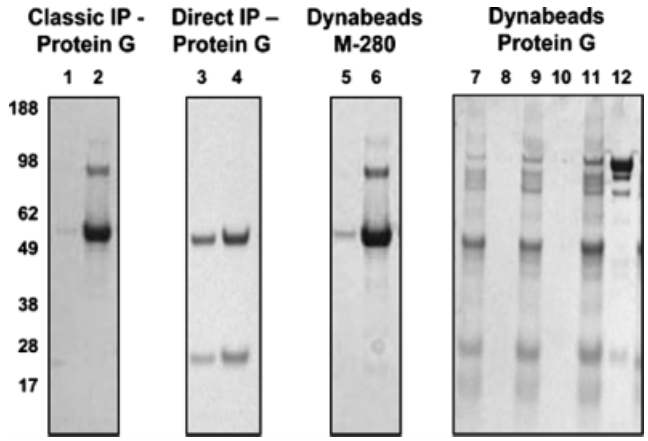


**Figure 3.** Coomassie-stained SDS-PAGE gels are used to compare the ability of four commercial kits to crosslink R&D mAb 1067 to a solid support. The four gels are labeled above the corresponding kit. Staining in lanes 2, 4 and 6 represents antibody remaining in the buffer following the reaction. This is the fraction of the antibody that did not successfully crosslink to the solid phase. Successfully bound antibody, if present, stains in the odd lanes. For the first three gels (lanes 1–6), the crosslinking reaction was carried out with the manufacturer's suggested amount of solid phase and 10 μg of antibody. In the final gel (lanes 7–12) the amount of antibody was varied. For lane 7, 1.5 mg of Dynabeads Protein G was crosslinked with 10 ug of antibody. For lanes 9 and 11 the amount of antibody was increased to 20 and 50 ug. The even numbered lanes (8, 10 and12) show whether or not antibody remains in buffer. None is present in lanes 8 and 10, however with 50 ug of antibody some remains in the buffer (lane 12).

respectively. Lanes 8, 10 and 12 represent the antibody remaining after successfully crosslinked material is removed by binding to the Protein G coated magnetic bead. This figure indicates that the Protein G beads have an efficient and large capacity to bind this antibody. Excess, unbound antibody does not appear until lane 6 at the 50 μg level.

The second step in the method comparison involved the antigen capture by crosslinked beads/antibody. Figure 4 is a comparison of antigen capture using each kit and mAb 1067. The results with the Classic IP - Protein G kit are shown in lanes 1–4. It can be seen from lane 1 that greater than 90% of the hGH in the assay remains unbound and contains significant antibody interference. Additional hGH and antibody washed off and can be seen in lane 2. The overall signal for the eluted hGH is very weak and contains antibody. These results agree with that seen for the kit in Fig. 3; the crosslink efficiency and stability is poor and antibody in continually released into the fractions. As there is only a fraction of the original 10 μg of antibody remaining in the immunoprecipitation step the antibody capture is also very low.

The second blot in Figure 4 was produced by the Direct IP – Protein G kit. This kit is very similar to the Classic IP – Protein G kit above with the addition of a chemical crosslinker to improve

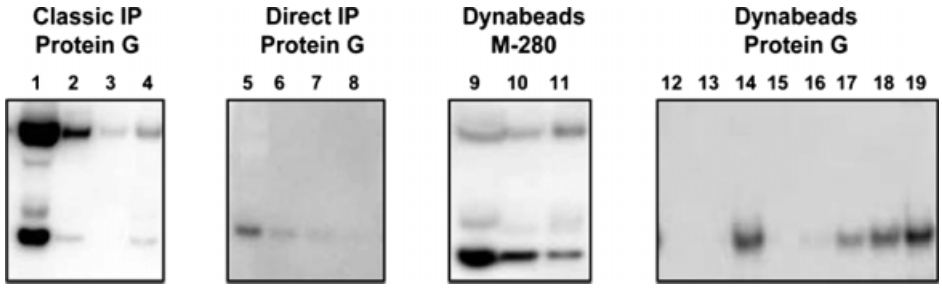


**Figure 4.** Antibody capture and elution comparison of each method using the R&D mAb 1067 by western blot. For all kits the immunoprecipitation (IP) was carried out according to manufacturer's suggested amount of solid phase and 10 μg of antibody. All fractions were resolved by SDS-PAGE followed by transfer to nitrocellulose and western blot. For each of the four blots the lanes are numbered. Lanes 1–4 represent the binding step supernatant, two washes and the elution fractions respectively for the Classic Protein G Kit. Lanes 5–8 are the same for the Direct IP Kit. Lanes 9–11 represent the binding supernatant, one wash and the eluate from the Dynabeads M-280 Kit. Lanes 12–15 represent the binding supernatant, wash, elution and used bead/antibody following the elution step. Lanes 16–19 represent a standard curve of 5, 25, 50 and 100 ng of native 22 kDa hGH for signal comparison and determination of capture-and-elution efficiency by densitometry.

the stability of the binding of the antibody to the Protein G on the solid phase. As expected, this resulted in improved crosslink efficiency (Figure 3, blot 2) and stability, as seen by the lack of antibody in the blot. This did not result in improved antibody capture. There remains significant hGH in the binding reaction (lane 5) and very little signal for the elution (lane 8). Increased crosslinking efficiency and stability appears to impede successful antibody capture.

Dynabeads M-280 were used in the experiment shown in lanes 9–11 of Figure 4. These three lanes represent the binding supernatant, wash and elution fractions of the experiment. Lane 9 indicates poor overall antibody capture as most of the hGH signal remains in the binding supernatant, and in agreement with the corresponding gel in Figure 3, poor crosslink stability. Nonspecific antibody signal is seen throughout the experiment. This instability leads to poor antibody capture and elution (lane 11).

The final blot (lanes 12–19) shows the results obtained with the Protein G Dynabeads kit. Lanes 12–15 represent the binding supernatant, wash, eluate and beads/antibody following elution, respectively. There is neither hGH nor antibody signal in lanes 12, 13 and 15, and this indicates the stability of the immunoprecipitation complex throughout the experiment, minimizing possible downstream suppression effects. hGH signal is found in lane 14 only. This indicates a successful antibody capture and elution.

As a measure of the ability to recover the hGH in the Dynabeads Protein G reaction we added a concentration curve of native 22 kDa hGH to this blot in lanes 16–19. Lanes 16–19 contained 5, 25, 50 and 100 ng respectively. The recovery of hGH for the Protein G Dynabeads was determined to be greater than 50% by standard densitometry measurement. The limit of detection by western blot for this method is approximately 5 ng. This indicates that the hGH present in the urine has been recovered and eluted successfully.

As these studies and comparisons evolved, the combination of poor or unstable crosslinking as shown by Coomassie-stained gels led to the exclusion of the polyclonal antibodies. As it became clear that the Protein G Dynabeads outperformed the other methods, the assay was optimized with that method. Both the R&D Systems mAb 1067 and the BiosPacific mAb A36020 performed well by our criteria, however, the final optimized experiment was carried out with the former due to uncertainties with the documentation obtained from BiosPacific.

## Discussion

The first goal was to develop a method to extract and concentrate hGH from human urine. In the second part of the study we intended to detect the hGH in the extract by mass spectrometry. As the project developed, it became apparent that the enrichment and immunoassay techniques would not be adequate to recover sufficient hGH from urine for subsequent mass spectrometric analysis. Even an Orbitrap mass spectrometer is not sufficient for this task without additional elements.

Here we have shown that 22 kDa hGH spiked into urine can be concentrated and recovered by centrifugal membrane separation with selective molecular weight cutoffs of 10 and 50 kDa. This ultrafiltration process removes much of the albumin and immunoglobulins, the major proteins found in urine. Under these conditions the urine is concentrated and enriched in hGH and >95% of the hGH is recovered.

The next step was to compare the performance of four commercially available immuno-precipitation kits that could be adapted to isolate hGH from urine. Five antibodies (two monoclonals and three polyclonals) were tested. Invitrogen's Protein G Dynabead kit in combination with R&D Systems mAb 1067 gives the highest yields of cross-linked hGH antibody to solid phase and it recovered more hGH (approximately 47%) than any of the other kits. The other kits produced lower recoveries due to inefficient and unstable cross-linking of the capture antibody to the solid phase. Further there was evidence that some antibodies were released during the hGH elution step. This reduced recovery and would have suppressed the hGH signal during mass spectrometry.

These data provide a general framework for discussing the feasibility of detecting hGH in urine. Considering three key factors, the overall hGH recovery of 45% described here, the estimate that an Orbitrap mass spectrometer might detect 0.2 ng of hGH, and urine hGH concentrations of 1 pg/mL, the test would require 400 mL of urine. In sports drug testing, the usual collection is about 100 mL. From these estimates it is evident that for the urine method to be successful, a method capable of achieving a much greater degree of hGH concentration is needed. Although this large urine volume is not compatible with routine doping control urine collection, experience with other tests shows that optimization is likely to decrease the required urine volume.

The high volume of urine required emphasizes the need for new and improved methods. In fact, a new method has been described: a recent report discusses the use of custom designed nanoparticles containing hGH 'bait' to extract and concentrate

hGH.[22] The hGH concentration was 0.175 pg/mL in one human urine. This is almost tenfold less than the prior estimates. Today there are no instrumental methods capable of achieving that level of detection. Any improved method must sufficiently enrich and distinguish between all hGH isoforms in a urine sample for any ratio and the effects of administered hGH on that ratio to be measured. The difficulty will be that for a method to be useful it must independently detect the 20 and 22 kDa isomers or it must provide a direct estimate of the ratio. Lastly the extraordinary degree of sensitivity and specificity of aptamers makes them ideal for an hGH project.[23] Furthermore an aptamer directed against hGH was very recently described.[24] It has not yet been developed into a test but that will likely come soon.

## Conclusions

One hundred nanograms of hGH was spiked into 20 ml of human urine and concentrated into approximately 100 µL with 95% recovery. From this concentrated sample approximately 50% can be recovered by immunoprecipitation using the Invitrogen Dynabeads Protein G kit. These recoveries are likely to improve with further effort and new technology, however they cannot currently supply sufficient 22 kDa isoform for quantitation by mass spectrometry. In addition, the amount of 20 kDa isoform is tenfold less, thus making the ratio method unfeasible in urine. Sufficient recoveries allowing the measurement of hGH isoform ratios may be possible using nanoparticles to enrich and aptamers to detect and distinguish.

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## References

[1] H. Liu, D. M. Bravata, I. Olkin, A. Friedlander, V. Liu, B. Roberts, E. Bendavid, O. Saynina, S. R. Salpeter, A. M. Garber, A. R. Hoffman, *Ann. Intern. Med.* **2008**, *148*, 747.
[2] J. S. Baker, M. R. Graham, B. Davies, *Eur. J Intern. Med.* **2006**, *17*, 479.
[3] R. Krysiak, A. Gdula-Dymek, A. Bednarska-Czerwinska, B. Okopien, *Pharmacol. Rep.* **2007**, *59*, 500.
[4] J. Powrie, A. Weissberger, P. Sonksen, *Drugs* **1995**, *49*, 656.
[5] M. R. Graham, B. Davies, A. Kicman, D. Cowan, D. Hullin, J. S. Baker, *Curr. Neurovasc. Res* **2007**, *4*, 9.
[6] O. Barroso, P. Schamasch, O. Rabin, *Growth Horm. IGF. Res.* **2009**, *19*, 369.
[7] M. Bidlingmaier, C. J. Strasburger, *Nat. Clin. Pract. Endocrinol. Metab* **2007**, *3*, 769.
[8] M. Bidlingmaier, Z. Wu, C. J. Strasburger, *J. Endocrinol. Invest.* **2003**, *26*, 924.
[9] J. D. Wallace, R. C. Cuneo, R. Baxter, H. Orskov, N. Keay, C. Pentecost, R. Dall, T. Rosen, J. O. Jorgensen, A. Cittadini, S. Longobardi, L. Sacca, J. S. Christiansen, B. A. Bengtsson, P. H. Sonksen, *J. Clin. Endocrinol. Metab.* **1999**, *84*, 3591.
[10] J. K. Powrie, E. E. Bassett, T. Rosen, J. O. Jorgensen, R. Napoli, L. Sacca, J. S. Christiansen, B. A. Bengtsson, P. H. Sonksen, *Growth Horm. IGF. Res.* **2007**, *17*, 220.
[11] A. T. Kicman, J. P. Miell, J. D. Teale, J. Powrie, P. J. Wood, P. Laidler, P. J. Milligan, D. A. Cowan, *Clin. Endocrinol. (Oxf.)* **1997**, *47*, 43.
[12] P. H. Sonksen, R. I. Holt, *Growth Horm. IGF. Res.* **2009**, *19*, 283.
[13] R. I. Holt, E. E. Bassett, I. Erotokritou-Mulligan, C. McHugh, D. Cowan, C. Bartlett, P. H. Sonksen, *Growth Horm. IGF. Res.* **2009**, *19*, 346.
[14] M. Thevis, W. Schanzer, *Analyst* **2007**, *132*, 287.
[15] T. Kuuranne, A. Thomas, A. Leinonen, P. Delahaut, A. Bosseloir, W. Schanzer, M. Thevis, *Rapid Commun. Mass Spectrom.* **2008**, *22*, 355.
[16] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, *Anal. Biochem.* **2002**, *311*, 119.
[17] L. H. Gam, S. Y. Tham, A. Latiff, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2003**, *792*, 187.
[18] A. Thomas, H. Geyer, M. Kamber, W. Schanzer, M. Thevis, *J. Mass Spectrom.* **2008**, *43*, 908.
[19] M. Saugy, C. Cardis, C. Schweizer, J. L. Veuthey, L. Rivier, *J. Chromatogr. B. Biomed. Appl.* **1996**, *687*, 201.
[20] L. Yang, H. Zhang, J. E. Bruce, *Analyst* **2009**, *134*, 755.
[21] E. P. Trieu, J. K. Gross, I. N. Targoff, *Methods Mol. Biol.* **2009**, *536*, 259.
[22] C. Fredolini, F. Meani, K. A. Reeder, S. Rucker, A. Patanarut, P. J. Botterell, B. Bishop, C. E. V. Longo, E. F. Petricoin III, L. A. Liotta, A. Luchini, *Nano. Res.* **2008**, *1*, 502.
[23] S. Tombelli, M. Minunni, M. Mascini, *Biosens. Bioelectron* **2005**, *20*, 2424.
[24] P. Calik, O. Balci, T. H. Ozdamar, *Protein Expr. Purif.* **2010**, *69*, 21.