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Short communication

Analysis of urinary human growth hormone (hGH) using hydrogel nanoparticles and isoform differential immunoassays after short recombinant hGH treatment: Preliminary results

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ABSTRACT

Successful application clinical-grade human growth hormone (hGH) immunoassays to the discovery of illegal doping cases has been rare. Indeed, the preferred biological matrix in doping control is urine, where the estimated baseline concentration of hGH falls well below the linear range and sensitivity threshold of all commercially available immunoassays, including hGH isoform differential immunoassays which can discriminate pituitary endogenous hGH from recombinant hGH. We employed hydrogel nanoparticles as a pre-processing step that concentrate urinary hGH into the linear range of isoform differential immunoassays.

We explored the characteristics of immunoassays in urine spiked with both phGH or rhGH, after pre-treatment with the nanoparticles. Subsequently, pre-treatment was applied to urine obtained from 3 healthy volunteers administered during three days with daily subcutaneous injections of 0.026 mg/kg/day rhGH, Genotonorm®.

Linearity between both rhGH and phGH concentrations in urine measured by a chemoluminescent assay (Immulite) and in the particle eluate was evident for differential immunoassays (R square higher than 0.999). In case of treated individuals the recombinant/pituitary concentration ratios remained above the established World Anti-Doping Agency (WADA) criterion for hGH misuse up to 24 h after the last administration dose, using both assays for volunteer 1 and 2 while in case of volunteer 3 results were inconclusive.

The use of nanoparticles appears to open the possibility of assessing rhGH misuse in urine.

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1. Introduction

Clinical-grade human growth hormone (hGH) immunoassays are of no use for application in doping control for a variety of reasons. Firstly, absolute levels of hGH have no value for doping control because of the multiplicity of factors affecting them [1,2]. Secondly, the physicochemical structural differences between pituitary hGH (phGH) and recombinant pharmaceutical preparation of 22 kDa (rhGH) require a detection strategy that specifically addresses this rhGH variant. Thirdly, the half-life of hGH in circulation following subcutaneous administration of rhGH is very short, between 15 (22 kDa hGH) and 19 min (20 kDa hGH) [3], which directly affects the time-frame to detect elevated hGH levels. Lastly, the preferred biological matrix in doping control is urine, where the estimated baseline concentration of hGH is roughly between 100 and 1000 times lower than in blood falling below the linear range and sensitivity threshold (50 pg/mL) of all commercially available immunoassays for hGH. Recently two sensitive luminescent immunoassays with preferential recognition of pituitary (phGH) or recombinant monomeric (rhGH) growth hormone have been developed and validated for serum samples [4]. The method is based on the use of two sandwich type immunoassays that preferentially recognize either mono- and oligomeric phGH variants

Abbreviations: Rec, recombinant; Pit, pituitary; hGH, human growth hormone; AAF, adverse analytical finding.

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Table 1
Concentration in urine samples spiked with rhGH or phGH after nanoparticles extraction as measured by Immulite and rec2/pit2 (Kit2) differential immunoassay.

Spiked urine samples rhGH (pg/ml)	Immulite hGH (pg/ml)	Kit 2 differential immunoassay rhGH (pg/ml)	Kit 2 differential immunoassay phGH (pg/ml)
274	272.50	235.95	119.30
27	13.28	26.50	16.75
7	2.35	4.05	4.70
3	2.15	2.75	3.35
phGH (pg/ml)	hGH (pg/ml)	rhGH (pg/ml)	phGH (pg/ml)
379	207.25	162.60	176.15
38	2.42	2.14	20.50
9	4.73	3.20	5.10
5	1.78	2.05	4.05

or the monomeric isoform of rhGH: rec1/pit1 (kit1) and rec2/pit2 (kit2). Assays were successfully used to detect elevated ratios in serum of dosed volunteers from 18 to 36 h, depending of gender and administered dose [4], after a single injection of rhGH. Although these isoform differential immunoassays have a low threshold values (LOQ less than 41 pg/mL), they are above the requirements for direct measurements in urine.

Consequently, the World Anti-Doping Agency (WADA) endorsed the differential immunoassay methodology for serum and released specific guidelines for the application of these hGH isoform assays for anti-doping analyses [5] leading to the unmasking of several cheating athletes.

An adverse analytical finding (AAF) is dependent on the two ratios calculated by dividing the concentration value (in ng/mL) obtained for rhGH by the concentration value for phGH in both assays and these figures must exceed decision limits established for the used kits: ratio1 for rec1/pit1 > 1.81 for males and 1.46 for females and ratio 2 for rec2/pit2 > 1.68 for males and 1.55 for females [5].

To apply these hGH isoform differential immunoassays to urine and overcome the issue of extremely low concentration of hGH, we employed hydrogel nanoparticles [6–10] as a pre-processing step that concentrate urinary hGH into the linear range of the rec1/pit1 and rec2/pit2 isoform differential immunoassays. We here present the preliminary results obtained by applying nanoparticles pre-treatment to urine samples spiked with pituitary and recombinant hGH and to urine samples from healthy volunteers administered with recombinant hGH.

2. Experimental

2.1. Materials

Among several bait functionalized hydrogel nanoparticles, Remazol Brillant Blue (RBB) nanoparticles (Ceres Nanosciences, Manassas, VA, USA), containing a small organic dye as bait, were employed to capture, preserve and concentrate hGH in urine, since as they were successfully employed in previous experiments [8]. Recombinant 22 kDa hGH Genotonorm® for the administration studies was purchased from Pfizer laboratories (Barcelona, Spain). Spike Recombinant 22 kDa hGH for spiking experiments was Humatrope® from Lilly pharmaceutical company (Indianapolis, USA) and pituitary hGH was a preparation from National Institute for Biological Standards and Controls [11]. GH immunoassay kits for Immulite were from Siemens-DPC (LA, USA). Two sandwich type immunoassays that recognize mono- and oligomeric phGH (rec1/pit1 – kit1) variants or the monomeric isoform of rhGH (rec2/pit2 – kit2) were provided by the CMZ Assay GMBH (Berlin, Germany). Rec is the immuno assay targeting preferentially the recombinant monomeric 22 kDa GH variant and Pit isimmuno assay targeting preferentially the pituitary derived GH variants. All reagents were of analytical-reagent grade purchased from Aldrich (Milan, Italy) and were used without further purification.

2.2. Biological samples

Spiked samples were prepared using urine from healthy volunteers added with four different concentrations of phGH: 379, 38, 9, and 5 pg/mL or four different concentrations of rhGH: 274, 27, 7, and 3 pg/mL.

Real samples were obtained from 3 healthy males administered during three days with daily subcutaneous injections of 0.026 mg/kg/day rhGH, Genotonorm® at time 0, 24 and 48 h.

The study was conducted in compliance with the "ethical principles for medical research involving human subjects" of the Helsinki Declaration, and the protocol was approved by the local ethical review board and authorized by the Spanish Agency for Drugs and Health Products (AEMPS, Madrid, Spain). Serum samples were collected at 0, 6, 12, 24, 30, 48, and 54 h while urine samples at 0 time and at the following intervals:, 6–10, 10–24, 30–34, 34–48, 48–54, 54–58, 58–72, 72–84, and 84–96 h. Spot urine samples from five additional healthy volunteers, not involved in the administration study, were also collected and processed. Details concerning these five subjects can be found elsewhere. All samples were stored at −20 °C until analysis.

2.3. Conditions and instrumentation

First we explored the characteristics of rec1/pit1 and rec2/pit2 immunoassays in urine samples spiked with both phGH or rhGH, after pre-treatment with nanoparticles. Twenty mL spiked urine were incubated with 2 mL functionalized RBB particles for 30 min as indicated by the manufacturer. Particles were isolated by centrifugation (19,000 rpm, 50 min, 18 °C) and washed twice with water. Proteins captured by the particles were eluted with organic solvents (1 mL acetonitrile–ammonium hydroxide, 70–30), split in two aliquots and lyophilized in presence of trehalose (1 mL for every 10 mL of urine sample processed) as pellet builder. One aliquot was reconstituted in 500 μL Immulite sample diluent and measured with Immulite-1000 (Siemens Corp., USA). The other aliquot was reconstituted in 500 μL sheep serum (Sigma–Aldrich Corp., St. Louis, MO, USA) and analyzed with the isoform differential immunoassays by an AutoLumat LB 953 Multi-Tube Luminometer (Berthold Technologies, Germany).

3. Results and discussion

Linearity between both rhGH and phGH concentrations in urine measured by Immulite and in the particle eluate was evident for rec2/pit2 immunoassay and the relationship could be mathematically described by linear equations with values of R squared higher

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