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High-Sensitivity Chemiluminescence Immunoassays for Detection of Growth Hormone Doping in Sports

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BACKGROUND: Recombinant human growth hormone (rhGH) is abused in sports, but adequate routine doping tests are lacking. Analysis of serum hGH isoform composition has been shown to be effective in detecting rhGH doping. We developed and validated selective immunoassays for isoform analysis with potential utility for screening and confirmation in doping tests.

METHODS: Monoclonal antibodies with preference for pituitary hGH (phGH) or rhGH were used to establish 2 pairs of sandwich-type chemiluminescence assays with differential recognition of rhGH (recA and recB) and phGH (pitA and pitB). We analyzed specimens from volunteers before and after administration of rhGH and calculated ratios between the respective rec- and pit-assay results.

RESULTS: Functional sensitivities were <0.05 μg/L, with intra- and interassay imprecision $\leq$8.4% and $\leq$13.7%, respectively. In 2 independent cohorts of healthy subjects, rec/pit ratios (median range) were 0.84 (0.09–1.32)/0.81 (0.27–1.21) (recA/pitA) and 0.68 (0.08–1.20)/0.80 (0.25–1.36) (recB/pitB), with no sex difference. In 20 recreational athletes, ratios (median SD) increased after a single injection of rhGH, reaching 350% (73%) (recA/pitA) and 400% (93%) (recB/pitB) of baseline ratios. At a moderate dose (0.033 mg/kg), mean recA/pitA and recB/pitB ratios remained significantly increased for 18 h (men) and 26 h (women). After high-dose rhGH (0.083 mg/kg), mean rec/pit ratios remained increased for 32 h (recA/pitA) and 34 h (recB/pitB) in men and were still increased after 36 h in women.

CONCLUSIONS: Using sensitive chemiluminescence assays with preferential recognition of phGH or rhGH, detection of a single injection of rhGH was possible for up to 36 h.

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Human growth hormone (hGH)[5] is abused in sports *(1, 2)*, although its performance-enhancing effects in healthy adults are unclear *(3–5)*. It is listed on the prohibited list *(6)* of the World-Anti-Doping Agency (WADA). Pituitary extracts (phGH) and recombinant preparations (rhGH) are available on the black market, and hGH has been found in the possession of athletes, trainers, and physiotherapists *(7, 8)*. No test is routinely included in antidoping controls, however.

Detecting hGH abuse was considered impossible because of specific physiological and physicochemical properties of the hormone, including pulsatile release, variable concentrations in normal subjects, amino acid and physicochemical similarity between phGH and rhGH, and short half-life in circulation *(7–10)*. Two different strategies have been proposed to overcome these difficulties.

The marker approach measures hGH-dependent proteins *(11)* and uses specific discriminant functions to calculate an index of the marker protein concentrations. The increase in this index after hGH administration exceeds physiological variability and fluctuations seen after exercise *(12, 13)*. Generating assay-specific reference ranges for each marker in appropriate populations remains challenging. Published data *(14)* confirmed that age and sex are the major determinants of variability, whereas ethnicity and sports type are less important *(15)*. After analysis of within-subject and analytical variability, a Bayesian model was developed to increase the reliability of a possible doping test based on measurement of the markers *(16)*.

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[5] Nonstandard abbreviations: hGH, human growth hormone; WADA, World Anti-Doping Agency; phGH, pituitary hGH; rhGH, recombinant hGH; mAb, monoclonal antibody; NIBSC, National Institute for Biological Standards; hGH-V, human placental growth hormone; hPL, human placental lactogen; hPRL, human prolactin; GHBP, growth hormone binding protein.

Table 1. Performance characteristics of the 4 assays.

	recA	pitA	recA/pitA	recB	pitB	recB/pitB
Analytical assay sensitivity, μg/L	0.022	0.028		0.021	0.023	
Functional assay sensitivity, μg/L	0.027	0.041		0.027	0.040	
Intraassay CV range, %	1.2–8.4	1.6–7.1	2.4–9.7	1.0–4.4	1.5–7.8	2.7–8.3
Interassay CV range, %	2.4–8.0	4.0–13.7	3.8–13.8	1.1–5.8	2.5–12.1	2.7–13.3
Linearity range, recoveries in %	87.8–108.9	89.8–109.3	91.0–107.0	89.4–110.8	86.6–110.9	87.6–112.5
Mean recovery rhGH, % (SD)	80.3 (6.1)	39.3 (9.8)		81.7 (8.8)	34.0 (10.8)	
Mean recovery phGH, % (SD)	92.3 (5.2)	89.2 (5.3)		91.1 (6.9)	90.2 (6.1)	

An alternative approach, the isoform approach, measures changes in the hGH isoform composition in circulation after rhGH injection *(17)*. Circulating hGH consists of several isoforms, 22-kDa hGH being most abundant, followed by 20-kDa hGH *(18–20)*. All isoforms also form dimers and multimers. In contrast, rhGH is monomeric 22-kDa hGH only. After rhGH administration, pituitary hGH release is suppressed, and monomeric 22-kDa hGH becomes predominant. Immunoassays have been developed based on monoclonal antibodies (mAbs) with preferential binding to either pituitary-derived hGH or rhGH preparations. Measuring 1 serum sample in 2 assays, one preferentially recognizing the 22-kDa isoform (rec assay), the another recognizing a variety of hGH isoforms (pit assay), allows the calculation of a rec/pit ratio, indicating the relative abundance of monomeric 22-kDa hGH. This ratio increases after rhGH administration *(9, 17)* and could be helpful in pharmacokinetic studies with rhGH, but also to detect rhGH doping in sports.

The world antidoping code and the international standards for laboratories *(21)* of WADA require an independent test to confirm any adverse analytical finding (positive case). For immunoassays, "independent tests" means antibodies recognizing independent epitopes. Accordingly, 2 sets of rec and pit assays were developed *(7, 22)*, which, based on in-house research reagents, were evaluated during the Athens (2004) and Torino (2006) Olympic Games *(23)*.

Because the isoform approach is based on the direct measurement of hGH, the window of opportunity for detection is determined by the short half-life of rhGH in circulation. The limit of quantification of the assays thus is a crucial parameter. Widespread application in doping analytics requires a validated, robust kit format for the assays. Here, we report the optimization of the limit of quantification and reproducibility of the 2 sets of rec and pit assays using chemiluminescence and coated-tube techniques. One set of assays could be used as a screening test, the other for confirmation of the results. Furthermore, we investigated the suitability of the improved assays for detection of previous administration of rhGH.

Materials and Methods

SELECTION AND CHARACTERIZATION OF ANTIBODIES

mAbs were generated in mice immunized with recombinant or pituitary-derived hGH preparations and selected with respect to their affinities to 22-kDa rhGH [88/624; National Institute for Biological Standards (NIBSC)] or phGH (80/505; NIBSC). We identified 2 mAbs exhibiting high affinities for 22-kDa rhGH (AK568 and AK566) and 2 with high affinity for phGH but low affinity for rhGH (AK567 and AK565). We used the 4 mAbs as capture antibodies in the 4 assays described in this article. Another mAb (AK569) was found to bind both rhGH and phGH with similar affinity and therefore could be used as detection antibody in combination with all 4 capture antibodies. Epitopes of all mAbs were identified by competitive binding experiments with a large spectrum of hGH variants and fragments (for details, see Supplemental Text 1, Supplemental Table 1, and Supplemental Fig. 1A–E, which accompany the online version of this article at www.clinchem.org/content/vol55/issue3).

ASSAY METHODS

Purified mAb AK569 labeled with Acridinium-NHS-Ester (in.vent Diagnostica GmbH) served as tracer mAb in all assays. Polystyrene tubes (Greiner) were coated with AK568 (recA) and AK567 (pitA) or AK566 (recB) and AK565 (pitB). Assays were calibrated with pituitary-derived hGH (80/505; NIBSC) diluted in sheep serum (Sigma Aldrich). We used human serum (BRAHMS) spiked with phGH (negative control) or a mixture of phGH and rhGH (positive control) for control samples.

For the assays, 50 μL calibrators, controls, and samples were incubated with 150 μL sample incubation buffer in the respective coated tubes (recA/pitA and recB/pitB) for 2 h at room temperature. After

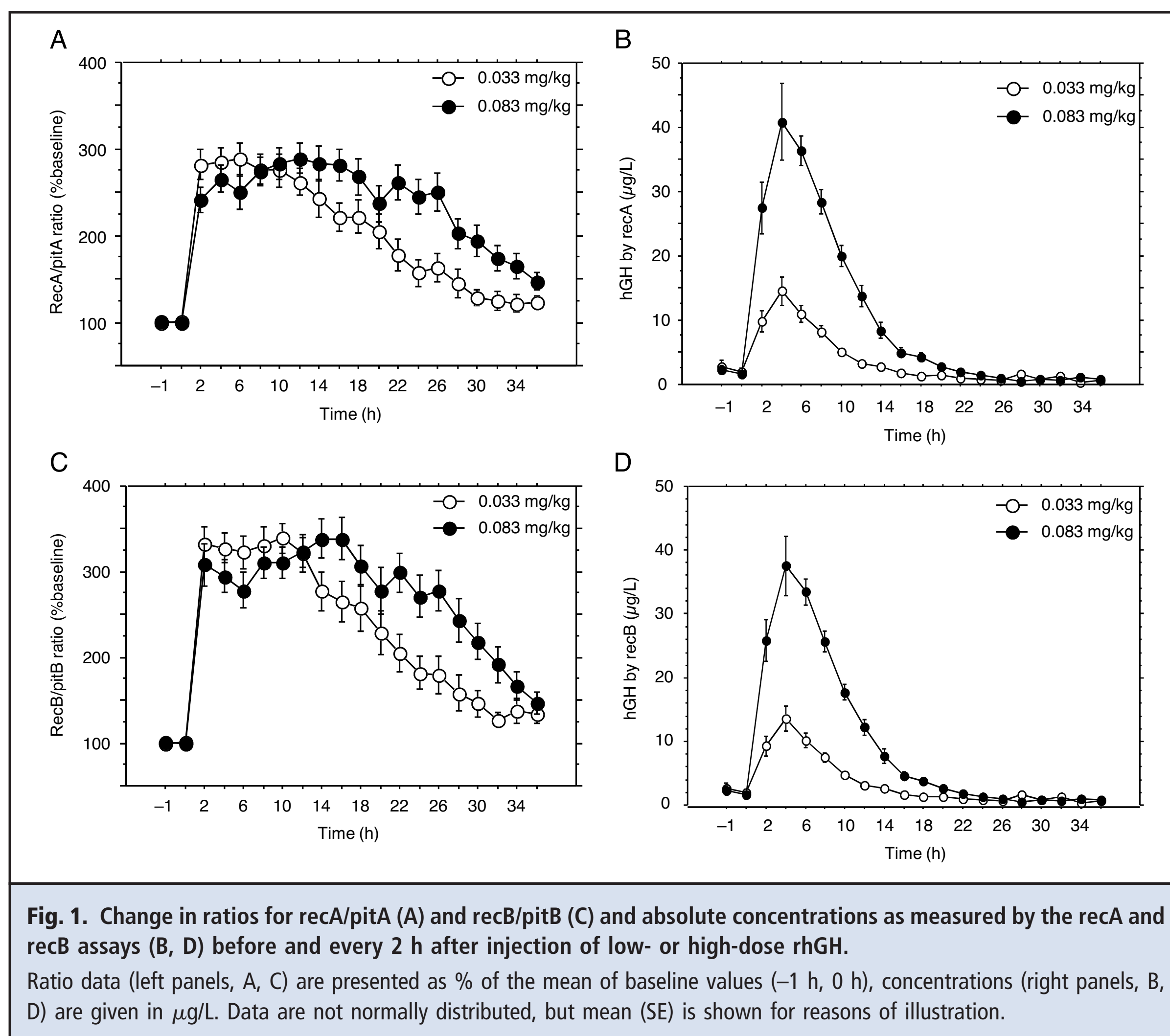

Fig. 1. Change in ratios for recA/pitA (A) and recB/pitB (C) and absolute concentrations as measured by the recA and recB assays (B, D) before and every 2 h after injection of low- or high-dose rhGH.

Ratio data (left panels, A, C) are presented as % of the mean of baseline values (–1 h, 0 h), concentrations (right panels, B, D) are given in μg/L. Data are not normally distributed, but mean (SE) is shown for reasons of illustration.

washing 5 times with 1 mL washing solution (8 mmol/L Tris, 0.06 mol/L NaCl, 0.02% Tween 20, 0.0002% defoaming agent), 200 μL tracer (AK569) was added and the tubes incubated for 2 h. After a final wash, we added acidic H_2O_2 and NaOH solutions (Basiskit reagents 1 and 2; BRAHMS) and measured the light signal using a luminometer (BeriLux Analyzer 250 or AutoLumat Plus LB 953; Berthold).

We also analyzed a subset of samples using the Immulite 2000 hGH assay (DPC/Siemens Medical Solutions) to provide comparative data with a commercially available method. Within-assay CVs were 3.2%, 2.3%, and 2.3%, and between assay CVs were 3.0%, 7.4%, and 2.3% at concentrations of 0.2, 2.1, and 27.1 μg/L, respectively. Functional sensitivity was 0.2 μg/L.

ASSAY VALIDATION

Sources of variants and fragments of hGH and other peptide hormones used, composition of buffers, and detailed procedures are listed in online Supplemental Text 2.

Analytical sensitivity was calculated as the concentration corresponding to the mean of a 10-fold measurement of the zero standard plus 2SD. Functional sensitivity is frequently defined as the lowest concentration that can be measured with an interassay CV $<$20%. However, none of the assays exhibited interassay CVs $>$20%, even for the background measurement. Therefore, we calculated the concentration corresponding to twice the mean signal of the zero standard from 10 independent runs. Intraassay CV was determined by 10-fold measurement of 20 samples at hGH concentrations (by assay pitA) ranging from 0.04 to 4.04 μg/L. Interassay CVs were determined from 20 samples (hGH 0.04–4.05 μg/L by assay pitA) analyzed in 10 independent assays by 3 operators on 5 days. Linearity was calculated from 5 samples (hGH range 1.3–9.6 μg/L by assay pitA) serially diluted in sheep serum

(1:2–1:16). Recovery was determined by adding different concentrations of rhGH (final concentrations 0–34.8 μg/L by recA) or phGH (0–40.0 μg/L by pitA) to aliquots of 5 samples, respectively.

In addition to mapping the epitopes for each antibody, we investigated cross-reaction of related proteins in the sandwich assays, where antibodies are combined. Potentially cross-reacting proteins were identified using a protein sequence database with protein–protein BLAST software. High degrees of homology were found for placental growth hormone (hGH-V, 178/191 amino acids), 20-kDa hGH (176/191), placental lactogen (hPL, 163/189), 17-kDa hGH 44–191 (148/191), prolactin (hPRL, 45/191), and hGH 1–43 (43/191). Sheep serum was spiked with these proteins at concentrations up to 500 μg/L (20-kDa hGH, 17-kDa hGH, hGH-V, hPRL) or 5000 μg/L (hGH 1–43, hPL) and measured using the 4 assays.

SUBJECTS AND SAMPLES

We obtained serum, EDTA plasma, heparin plasma, and citrate plasma from 9 healthy subjects to study the influence of anticoagulants.

Ten healthy volunteers provided blood samples from which serum was obtained for the investigation of preanalytical stability of the analyte. Aliquots from 10 volunteer samples were stored at room temperature (2, 4, 6, and 24 h), at 2–8 °C (6, 24, 48 h and 7 days), and at −15 °C (7, 30, 180 days). The reference aliquot was centrifuged after clotting and analyzed immediately. In addition, a set of aliquots was subjected to 4 freeze/thaw cycles before analysis.

To investigate the distribution of rec/pit ratios in untreated subjects, we collected samples from 2 independent cohorts of healthy blood donors (Berlin and Bavaria Red Cross) (serum tubes with separator gel; Monovette). We analyzed additional leftover serum samples from a study in recreational athletes (10 men, 10 women) receiving single injections of rhGH at different doses (moderate dose, 0.033 mg/kg; high dose, 0.083 mg/kg). Details of the protocol and participants of this study have been published *(24)*. In 24 samples from this study, hGH concentrations in the pitA and/or pitB assay were below the functional sensitivity. For statistical analysis, these samples were arbitrarily set to 0.05 μg/L.

Study protocols were in accordance with the current revision of the Helsinki Declaration and approved by the local ethics committees (Medical Faculties, Ludwig-Maximilians-University, Munich, and University of Leipzig). All subjects gave informed consent.

STATISTICAL ANALYSIS

We calculated concentrations of hGH in samples by relating the measurement signal to the signals obtained for the standard curve using the Mulitical software (spline smoothed algorithm, Wallac 1224–310 Multicalc, version 2.7; PerkinElmer). Identical results were obtained with the LBIS software (LB2111-LBIS; Berthold).

The mean (SD) or median (95% CI) were calculated. We compared results from the Immulite and the new assays using Deming regression analysis (Analyze-It Clinical Laboratory, V1.67; Analyze-It Ltd.). Further statistical analysis used Statview 5.0 (SAS Institute). We used nonparametric Mann–Whitney *U*-test for between-group comparisons (sex and dose) and compared data before and after injection of rhGH in the administration study using Wilcoxon signed-rank test. $P < 0.05$ was considered significant.

Results

EPITOPE MAPPING OF ANTIBODIES

Cross-reactivity of the respective mAbs with different variants and fragments of hGH is shown in online Supplemental Table 1. Epitopes of antibodies AK566 and AK568 partially overlap with binding site 1 for the hGH receptor, as they compete with growth hormone binding protein (GHBP) for binding of hGH, whereas epitopes for AK569, AK567, and AK565 are located distant from this site. AK569 does not bind 20-kDa hGH, indicating an epitope involving amino acid residues 32–46, which are spliced off in the smaller isoform. AK565 binds the 17-kDa hGH variant, lacking amino acids 1–44. Thus, the epitope cannot be on helix 1. AK567 and AK565 have reduced affinities for the variant G120K (25% and 37.5%, respectively), suggesting epitopes close to amino acid 120. Epitopes of the mAbs deduced from these experiments are plotted on a structural model of hGH in online Supplemental Fig. 1A–E.

SENSITIVITY, PRECISION, LINEARITY, RECOVERY, AND CROSS-REACTIVITY

The performance characteristics for the 4 assays are summarized in Table 1.

No cross-reactivity was found for 20-kDa hGH, hGH 1–43, hPL, and prolactin. At 100 μg/L, 17-kDa hGH exhibited 2.8% (recA), 0.4% (pitA), 0.7% (recB), and 0.2% (pitB) cross-reactivity, whereas hGH-V showed 14.1% (recA), 28.0% (pitA), 16.1% (recB), and 17.6% (pitB) cross-reactivity. Rec/pit ratios for sheep serum spiked with 10 μg/L hGH-V were 0.51 (recA/pitA) and 0.60 (recB/pitB).

INTERFERENCE

No interference from hemoglobin (5 g/L), bilirubin (684 μmol/L), triglyceride (39.9 mmol/L), or human serum albumin (10 g/L) was observed in any of the assays. In 8 serum samples spiked with recombinant hGHBP (220–3520 pmol/L), measured concentrations

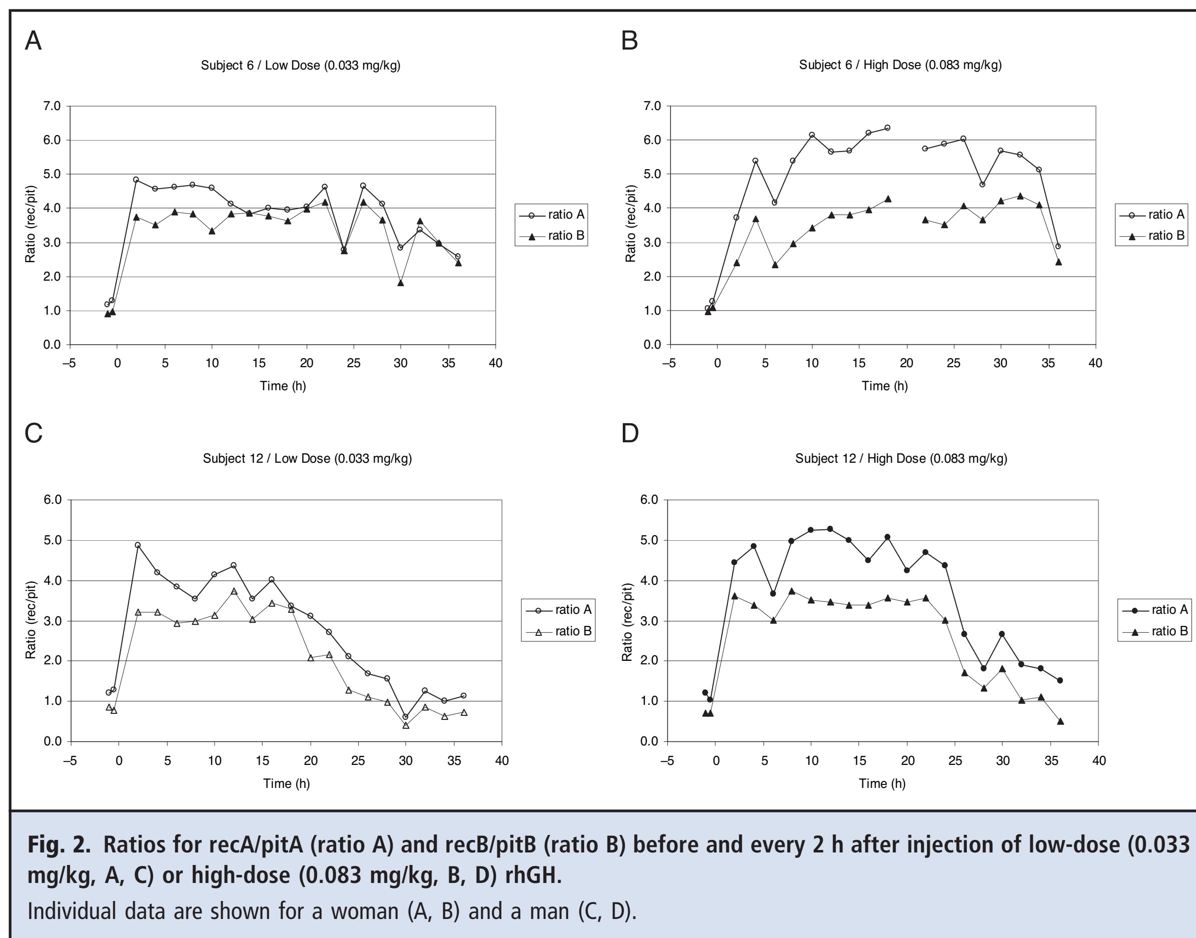

Fig. 2. Ratios for recA/pitA (ratio A) and recB/pitB (ratio B) before and every 2 h after injection of low-dose (0.033 mg/kg, A, C) or high-dose (0.083 mg/kg, B, D) rhGH.
Individual data are shown for a woman (A, B) and a man (C, D).

of hGH declined with increasing hGHBP in all assays. At the highest hGHBP concentration, hGH concentrations were reduced to 48.4% (4.9%) (recA), 63.9% (5.5%) (pitA), 55.3% (4.3%) (recB), and 60.2% (6.9%) (pitB), respectively. Rec/pit ratios remained unaffected (variation <10%).

Compared to the hGH concentrations in 9 serum samples (hGH 0.03–5.58 μg/L by assay pitA), the concentrations determined in the corresponding EDTA-, heparin-, and citrate-plasma samples were slightly higher: 108.6% (4.7%), 109.3% (5.9%), and 113.4% (6.6%) for recA, 119.9% (8.4%), 107.8% (9.9%), and 111.4% (6.9%) for pitA, 113.4% (6.6%), 112.4% (6.6%), and 116.3% (5.7%) for recB and 118.4% (7.7%), 111.3% (9.5%), and 111.2% (7.6%) for pitB, respectively. The rec/pit ratios corresponded to 90.8% (7.2%), 102.0% (8.8%), and 102.1% (7.8%) of the ratios obtained in serum for recA/pitA and 96.4% (3.9%), 101.2% (3.8%), and 104.9% (6.2%) for recB/pitB.

ANALYTE STABILITY

Neither the absolute hGH concentrations nor corresponding rec/pit ratios changed in any of the assays up to 24 h at room temperature or at 2–8 °C. After storage at 2–8 °C for 48 h or 7 days, absolute concentrations in pitB tended to decrease and mean recB/pitB ratios increased [108.5% (5.8%) and 110.2% (5.0%)], whereas concentrations in all other assays and recA/pitA ratios remained unchanged. After 180 days at −15 °C, mean absolute concentrations for assays pitA and pitB were 10% lower compared to baseline, and recA/pitA and recB/pitB ratios corresponded to 115.0% (7.9%) and 108.8% (4.8%) (online Supplemental Fig. 2A–C). All changes failed to reach statistical significance, however. Neither the concentrations nor the calculated ratios changed significantly with freeze/thaw cycles (online Supplemental Fig. 2D).

ANALYSIS OF HUMAN SERUM SAMPLES

Healthy volunteers (blood donors). Concentrations of hGH were measured using the recA and pitA assays for 100 serum samples from blood donors. In 24 samples, hGH was below the functional sensitivity of the pitA assay. In the remaining 76 samples (28 men/48 women), concentrations of hGH as measured by pitA

Table 2. Analysis of hGH concentrations and rec/pit ratios in 2 independent cohorts of blood donors.

	hGH, μg/L		recA/pitA ratio	hGH, μg/L		recB/pitB ratio
	recA	pitA		recB	pitB	
Cohort 1[a]						
Minimum	0.05	0.06	0.09	0.05	0.06	0.08
25th percentile	0.08	0.12	0.64	0.08	0.13	0.50
Median	0.41	0.53	0.84	0.19	0.46	0.68
75th percentile	1.53	1.85	0.97	0.92	1.38	0.87
Maximum	15.10	17.90	1.32	8.82	8.88	1.20
Lower 95% CI of mean	0.83	1.01	0.76	0.40	0.65	0.63
Upper 95% CI of mean	2.11	2.46	0.87	1.27	1.62	0.76
Cohort 2, n = 100						
Minimum	0.10	0.11	0.27	0.10	0.12	0.25
25th percentile	0.15	0.25	0.68	0.16	0.29	0.62
Median	0.44	0.57	0.81	0.48	0.66	0.80
75th percentile	1.09	1.43	0.91	1.17	1.71	0.90
Maximum	9.24	10.10	1.21	9.65	11.70	1.36
Lower 95% CI of mean	0.72	0.88	0.75	0.79	0.99	0.72
Upper 95% CI of mean	1.33	1.56	0.82	1.43	1.73	0.80

[a] n = 76 for assay A and n = 50 for assay B.

ranged from 0.06 to 17.90 μg/L (median 0.53 μg/L). Because of limited volume, only 50 samples were also measured by pitB and recB assays, and the respective rec/pit ratios were calculated (Table 2). Mean ratios did not differ between men and women (rec/pitA, $P = 0.80$; rec/pitB, $P = 0.86$).

In an independent cohort of blood donors, hGH was measured by the Immulite assay first, and only 100 samples with measurable hGH concentrations (≥0.2 μg/L; median 0.80 μg/L, range 0.20–16.80 μg/L) were selected [34 men/66 women, mean (SD) age 42.9 (13.9) years, range 19–69 years]. In pitA, hGH concentrations ranged from 0.11 to 10.1 μg/L (median 0.57 μg/L). Corresponding results for the other assays and rec/pit ratios are shown in Table 2. The ratios did not differ between sexes (rec/pit A, $P = 0.84$; rec/pitB, $P = 0.61$) and were independent of subject age or absolute hGH concentration ($P > 0.05$).

The Immulite assay results were significantly higher than those obtained by either of the differential immunoassays [Deming regression analysis: $P < 0.0001$, slope 0.56 ($r = 0.933$) for recA, 0.62 ($r = 0.98$) for pitA, 0.60 ($r = 0.99$) for recB, 0.68 ($r = 0.98$) for pitB].

Administration study in recreational athletes. A single rhGH injection of 0.033 or 0.083 mg/kg in 10 men and 10 women *(24)* led to a significant increase in the rec/pit ratios within 2 h. Peak recA/pitA ratios reached 350% (73%) compared with baseline, with no difference between sexes ($P = 0.378$) or dose groups ($P = 0.685$). Peak recA/pitA ratios were observed 12.0 (7.7) h after injection (range 2–30 h), and mean time between injection and peak ratio tended to be longer at the higher dose [9.9 (7.0) h vs 14.1 (8.0) h, $P = 0.086$]. RecB/pitB ratios increased to 400% (93%) of the baseline ratios 13.0 (7.9) h postinjection (range 2–32 h), with no significant difference between sexes. Again, mean time between injection and peak ratio tended to be longer at the higher dose [10.7 (6.2) h vs 15.3 (8.8) h, $P = 0.061$]. The time course of group means of the ratios after moderate- and high-dose rhGH, as well as the corresponding hGH concentrations as measured by recA and recB, are shown in Fig. 1A–D.

Mean recA/pitA ratios remained above baseline for 18 h in men ($P = 0.028$) and 26 h in women ($P = 0.010$) after the 0.033 mg/kg (moderate) rhGH dose. Corresponding recB/pitB ratios remained significantly increased for 18 h in men ($P = 0.018$) and 26 h in women ($P = 0.003$). After the higher 0.083 mg/kg dose, the mean recA/pitA ratio remained significantly increased for 32 h in men ($P = 0.028$) and was still increased after 36 h in women ($P = 0.010$). The corresponding mean recB/pitB ratio remained significantly higher for 34 h in men ($P = 0.018$) and was still in-

creased after 36 h in women ($P = 0.026$). The magnitude and duration of the increase varied significantly between subjects. For illustration, data from 2 individuals at both dosages are shown in Fig. 2A–D.

Discussion

We present a set of differential immunoassays for the detection of previous rhGH administration, combining selective monoclonal antibodies with an analytically sensitive chemiluminescence technology in a coated-tube format.

The principle of the differential immunoassay approach has been described *(9, 17)*, and an in-house version of the assays was evaluated during the Olympic Games in Athens 2004 and Torino 2006 *(23)*. The fact that no adverse analytical findings were reported might indicate that athletes were not taking hGH or that it was used during training rather than competition. It is also obvious, however, that the in-house version of the assays, using nonroutine research reagents and microtiter plates coated on site, showed poor performance and robustness. The sensitivity of 0.1 μg/L reported when the assays were initially developed *(25)* could not be routinely maintained after transfer of reagents and operating instructions to antidoping laboratories. Therefore, we aimed to develop a standardized, robust, and extremely sensitive format of the assays. The 4 assays now all have functional sensitivities <0.05 μg/L and are reproducible. Variability of calculated rec/pit ratios was <14% even when different operators performed the assays on different days.

As expected, the pit assays, employing antibodies specifically selected for preferential binding to pituitary-derived hGH, exhibit greatly reduced recovery of rhGH. In contrast, the recovery of phGH was comparable in all 4 assays. This is a confirmation of the principle behind the differential immunoassay approach, but also relevant in view of the ongoing debate on hGH assay standardization *(26)*. Both the calibrators and epitope specificity of antibodies have tremendous impact on immunoassay results. In clinical routine diagnostics, this leads to problems regarding comparability of assay results and application of published cutoffs for dynamic tests in GH-related disorders *(27)*. In contrast, in the assays presented here, the discrepancy in the affinity of the antibodies for different hGH isoforms is intentionally used to establish the origin of hGH found in circulation.

The only significant cross-reactivity of hGH-related molecules in the assays was observed for hGH-V, the placental variant of hGH *(28, 29)*, and occurred at concentrations >10 μg/L. In single pregnancies, hGH-V normally does not exceed 5 μg/L during the first months of gestation. Therefore, no consequences can be expected in a doping detection setting in female athletes with early pregnancy. End-stage twin pregnancies, where hGH-V might reach 100 μg/L, do not reflect the normal conditions of female athletes being tested.

A negative bias for hGHBP has been reported for several commercially available hGH assays *(30, 31)* and was also observed in the differential immunoassays. Reduction in the signal occurred for all 4 assays, and the rec/pit ratios obtained remain unaffected. The 20-kDa hGH isoform cross-reacts with all capture mAbs but does not interfere in any of the 4 sandwich assays, because the epitope recognized by the labeled antibody AK569 includes amino acids missing in the 20-kDa isoform. This has implications for future developments of other methods to detect hGH doping—because injection of rhGH leads to suppression of the 20-kDa hGH isoform *(24, 32)*, immunoassays specifically measuring 20-kDa hGH could be used as an independent measure to confirm rhGH administration.

The analytical and functional sensitivities of our assays are below those achieved with most commercially available routine assays. Increased sensitivity and improved reproducibility allow reliable calculation of rec/pit ratios even at low hGH concentrations. Rec/pit ratios were independent from absolute hGH concentration, identical in 2 independent cohorts of untreated subjects, and not different between sexes. Rec/pit ratios significantly increased within 2 h after a single injection of rhGH, and peak ratios corresponded to 300%–400% of the baseline ratios for both assay pairs. Results from the rhGH administration study suggest that the window of opportunity for detection is variable between subjects, ranging from 10 h to beyond 36 h. It is longer at a higher dose and longer in females than in males. The increase in the ratio mainly depends on 2 factors, the presence of rhGH in circulation and the reoccurrence of phGH secretion after initial suppression. The kinetics of hGH release from the subcutaneous depot *(24)* and the disappearance from circulation are variable between individuals *(33)*, explaining the variability of window of opportunity. The impact of repeated rhGH dosing was not tested in this study, but should be investigated to develop an effective strategy for antidoping testing.

Application of the differential immunoassays in antidoping laboratories would require definition of a cutoff above which the administration of rhGH can be assumed with reasonable confidence. Definition of such a cutoff was beyond the scope of our studies, and is a task for authorities and organizations involved in doping controls. It must be based on ratios obtained from a suitable reference population, where samples are taken under conditions comparable to those occurring in antidoping controls. Furthermore, although

concentrations and ratios were not significantly changed by any of the preanalytical conditions we tested, these conditions might differ from those used in an antidoping test scenario, and stability in a real-life scenario should be investigated.

In summary, we describe a set of 4 robust and sensitive chemiluminescence immunoassays for the measurement of hGH, which in combination through their differential recognition of hGH isoforms allow reliable detection of rhGH use. They can potentially be useful to monitor the pharmacokinetics of rhGH or compliance of treated patients, but also to detect abuse of rhGH in sports. In some individuals, a change in the isoform composition can be detected for more than 36 h, but owing to the short half-life of hGH, the likelihood to detect rhGH administration is highest during the first 24 h.

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