Influence of Matrix on Concentrations of Somatotropin Measured in Serum with Commercial Immunoradiometric Assays

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Using immunoradiometric assays (IRMAS) from Hybritech Inc. (H) and Nichols Institute Diagnostics (ND), we measured somatotropin (human growth hormone, hGH) in serum samples obtained every 20 min for 24 h from 10 prepubertal subjects with short stature. Results obtained with the ND reagents were 2.74 times greater than those obtained with the H reagents (P = 0.00001, r = 0.94, SEE = 3.9, n = 720). We therefore compared the IRMAs with the standard hGH RIA from the National Institutes of Health (NIH) National Hormone and Pituitary Program, using the genetically engineered hGH preparations (from Genentech Inc.) 22-kDa hGH and methionated 20-kDa hGH. We also assayed human pituitary hGH (NIH, lot no. AFP-4793B). Each hGH preparation was diluted in three diluent buffer systems: horse serum from H and from ND, and human serum. The RIA and H-IRMA gave superimposable standard curves for all hGH preparations in each diluent. The methionated 20-kDa hGH was not detected in the H-assay. Use of human serum matrix in the ND-IRMA shifted the standard curve as compared with the horse-serum matrix, giving equivalent binding at lower concentrations; i.e., serum hGH was overestimated in samples assayed against standards diluted in horse serum. Quality-control materials (Ciba-Comin) yielded disparate results in all three assays, yet human serum pools containing hGH gave similar results in the H and the NIH assays, and higher values in ND. When a human serum standard was used in the ND assay, both IRMAs gave similar results to the RIA assay for human serum samples. Reference intervals for hGH should be determined by each analytical laboratory, to prevent misdiagnosis of patients. Furthermore, quality-control material should be of human origin, because commercially supplied quality-control material does not react the same as human serum in some hGH assays.

Additional Keyphrases: variation, source of standardization, radioimmunoassay compared

Somatotropin (human growth hormone; hGH), a polypeptide hormone synthesized in the anterior pituitary, has a wide variety of effects on growth and metabolism. The diagnosis of hGH deficiency relies on the accurate estimation of hGH concentrations in serum, either in the basal state or after provocative stimuli. Basal hGH concentrations are assessed by comparing the results with an appropriate reference interval. Reference intervals are not always established by each clinical laboratory that reports hGH concentrations; however, patients' results are sometimes compared with published values, which may have been derived from different immunoassay systems. Differences as great as 65% have been previously reported for hGH concentrations in serum as measured by two different commercial assays (1). Furthermore, hGH concentrations in pediatric patients with hGH-dependent growth failure differed by 60% when measured by immunoradiometric assay (IRMA) and radioimmunoassay (RIA) (2), discrepancies that could lead to misdiagnosis.

Using IRMAs from two commercial sources, we measured hGH in serum obtained every 20 min for 24 h from 10 prepubertal subjects with abnormally short stature. The difference in hGH concentrations as measured by the two assays was almost threefold. We therefore further examined samples for hGH concentrations in three assay systems: the two IRMAs and an RIA. We measured three molecular forms of hGH in three different serum matrices, as used in the commercial immunoassays.

Materials and Methods

Assays. We used three different immunoassay methods to quantify hGH. Our reference assay was an RIA, the components of which were obtained from the National Institutes of Health (NIH) National Hormone & Pituitary Program (National Institute of Arthritis, Diabetes and Digestive & Kidney Disease, Bethesda, MD). This assay is a double-antibody precipitation assay, with goat antirabbit antiserum to separate bound antigen from free radiolabeled antigen. The NIH standard (lot no. AFP-4793B) consists of hGH extracted from cadaveric pituitaries. We dissolved the standard in, and diluted it with, hGH-free human serum, then stored it in aliquots at −80 °C. Human serum that was negative for hGH by all three assays was pooled and used as hGH-free human serum. The assay was performed as previously described (3). The detection limit was 0.25 μg/L, determined as the value exceeding 2 SD from the mean of 30 replicate analyses of the zero calibrator. Inter- and intra-assay coefficients of variation (CVs) were 5.6% and 5.1%, respectively, at an hGH concentration of 25.0 μg/L (n = 30).

The second assay (H-IRMA) was the Tandem-hGH from Hybritech Inc., San Diego, CA. The assay is configured as a double monoclonal antibody solid-phase IRMA. The antigen is incubated in the presence of an antibody-coated bead and a separate, radiolabeled antibody that is directed at another epitope on the hGH molecule. Bound and free labeled antibody are separated by washing. The calibrator consists of purified hGH in horse serum, referenced to the NIH reference preparation HS-2243E (National Hormone & Pituitary Program). We performed the assay according to the manufacturer's instructions, without modification. The detection limit of the assay was less than the 0.2 μg/L used as a cutoff value in our experiments. Inter- and intra-assay CVs were 4.9% and 4.2%, respectively, at an hGH concentration of 25.0 μg/L (n = 30).
We also used the "Allegro hGH" from Nichols Institute Diagnostics (San Juan Capistrano, CA), a double monoclonal antibody IRMA, with one antibody linked to biotin and the other radiolabeled (N-IRMA). The ternary complex is exposed to an avidin-coated bead to separate bound from free antibody. The assay was calibrated with purified hGH from NIH (NIAMDD-hGH-RP-1) and the World Health Organization (First International Reference Preparation, 1st IRP 66/217) in horse serum, and was performed according to the manufacturer's instructions, without modification. The assay detection limit was approximately 0.06 μg/L. Intra- and interassay CVs were 3.4% and 4.3%, respectively, at an hGH concentration of 25.0 μg/L (n = 30).

Results for all assays were quantified in a Micromedic gamma counter equipped with automated data reduction ("RIA-AID", Robert Maciel, Arlington, MA). A log-log standard curve linearization method was used for the NIH-RIA. The N-IRMA and H-IRMA standard curves were linearized with a four-parameter logistic transformation. Data were accepted for all analytical runs in which results for quality-control samples were within 2 SD of the mean established from our previous 20 analytical runs.

Statistical analysis. For comparison of immunoassays, we used Deming's regression (4). The concentrations of hGH in control material were compared by using Student's (two-tailed) t-test. Analysis of variance followed by Duncan's test was used after log transformation of the standard curve data to determine the significance of the differences between assays.

Subject groups. Specimens from 10 children were obtained under institutionally approved protocols. We collected 720 specimens from 10 children who were undergoing evaluation for idiopathic short stature, a specimen being collected every 20 min for 24 h. Thirty-two additional specimens were selected at random from specimens collected at a local health fair. These additional specimens were early-morning samples and were obtained after informed consent.

Standards. We suspected that the analytical differences in hGH quantification were attributable to different matrices (5). We therefore examined the ability of all three assays to measure three forms of hGH: hGH extracted from human pituitaries, obtained from the National Hormone & Pituitary Program; and recombinant methionated 20-kDa GH and the natural sequence, 22-kDa GH, obtained as gifts from Genentech (South San Francisco, CA).

Matrices. The ability of the three assays to quantify the three forms of hGH was examined in three different incubation matrices: (a) horse serum, obtained as a gift from Hybritech Inc., (b) horse serum, obtained as a gift from Nichols Institute Diagnostics, and (c) human serum stripped of hGH by use of monoclonal antibodies to hGH. This last serum was shown to have unmeasurable hGH concentrations in all three assays (n = 25).

Results

Figure 1 shows the results of the comparison of analytical results for hGH in normal subjects. hGH concentrations were 2.57 times greater as measured with the Nichols IRMA as compared with the Hybritech IRMA (n = 32, r = 0.98, SEE = 2.47). For the children who were examined for short stature we discovered 2.74 times greater hGH concentration in the N-IRMA as compared with the H-IRMA (n = 720, r = 0.94, SEE = 3.9) (Figure 1). Figure 2 shows a representative 24-h profile for hGH secretion in one patient. The almost threefold difference between the N-IRMA and H-IRMA was consistent throughout the duration of the pulses and at all concentrations, as was also illustrated by the good correlation throughout the range of the linear regression of the data (Figure 1).

We next measured hGH concentrations in commercially obtained (Ciba Corning, Irvine, CA) as well as pooled human serum quality-control material (Table 1). The lowest values in the commercially prepared quality-control material for hGH were obtained with the H-IRMA. RIA results were two to three times greater than those of the H-IRMA. The N-IRMA yielded approximately three times
greater results than the H-IRMA. Package-insert data obtained from Ciba-Corning showed hGH discordance as well (see Discussion).

hGH measured in the pooled human serum quality-control material, in contrast to the commercial material, showed good agreement between the H-IRMA and the RIA (P < 0.05), but N-IRMA values were about three times greater than those obtained with the other immunoassays (P < 0.05).

Fig. 3. hGH concentrations measured in three purified hGH preparations—pituitary extract hGH (a), 22-kDa GH (b), and methionated 20-kDa GH (c)—by the three immunoassays in two assay matrices: horse serum (▲), and human serum (●).

For each immunoassay, standards were prepared in the horse serum diluent supplied with that assay. All forms of hGH were measured similarly in the NIH and Hybritech assays except that 20-kDa GH was not detected by the Hybritech assay. The Nichols assay over-recovered hGH when calibrated with standards prepared in Nichols horse serum but not with standards prepared in human serum; (*) P < 0.05, analysis of variance, followed by Duncan's test (n = 3).

To determine the source of the discordance between the H-IRMA and the N-IRMA, we evaluated three different hGH preparations (extracted pituitary, recombinant natural sequence 22-kDa, and recombinant methionated 20 kDa) in two different matrices (human and horse serum). We included the RIA in these comparisons. Figure 3 shows data for the comparisons in human serum and horse serum observed with either the RIA, H-IRMA, or N-IRMA. Each assay was calibrated with manufacturer-supplied standards, both in manufacturer-supplied horse serum diluent and in human serum. The methionated 20-kDa GH was not detected in the H-IRMA (Figure 3c). The N-IRMA had a left shift in the standard curve, resulting in overestimate of serum GH when we used their standards diluted in Nichols horse serum (Figure 3 a–c, bottom panels). When a human serum standard was used in the N-IRMA, both IRMAs gave results similar to those of the NIH RIA for human serum samples (Table 1).

Discussion

Before the advent of IRMAs involving monoclonal antibodies, there was good agreement between results obtained by use of many commercial RIA reagents for quantifying hGH (6). However, Blethen and Chasalow (2) described lower "recoveries" of hGH in serum of children with GH-dependent growth failure when the H-IRMA was compared with a polyclonal RIA (the IRMA/RIA ratio was 0.48 for normal individuals). They suggested that this discrepancy was due to the presence of GH-like molecules that were being quantified by the polyclonal antisera but not in the highly specific two-site monoclonal IRMA. Levin et al. (7) measured a lower hGH concentration in serum by H-IRMA than by the NIH RIA (IRMA/RIA ratio 0.75). When the H-IRMA was compared with another monoclonal IRMA (Wellcome Co.) there was a greater discrepancy (H-IRMA/Wellcome IRMA ratio = 0.55). These authors concluded that the differences in hGH recovery between the assays studied was not ascribable to circulating molecular variants by hGH. Rose et al. (8) compared the H-IRMA with a commercial RIA and correlated the results with an in vitro bioassay. Values obtained with the Hybritech IRMA were approximately half of the concentrations obtained by RIA. The results obtained by RIA more closely approximated the results obtained by the Nb2 cell bioassay. These authors concluded that somatotropin variants with biological activity may be missed by the highly selective two-site IRMA. Thus there have been three independent observations of discordance between hGH immunoassays, but no unifying mechanism has yet been determined for the discrepancy.

Results of an evaluation of three different hGH preparations in a variety of matrices suggested that horse serum leads the N-IRMA to overestimate hGH. Horse serum did not interfere in either of the other assays. When a human serum matrix was substituted in the N-IRMA, all of the hGH standards compared favorably between assays. Furthermore, in both commercial and pooled human serum quality-control material, the N-IRMA formulated with
human serum standards gave similar analytical recoveries of hGH (Table 1, last column). Although we know that use of horse serum leads to erroneous results in the N-IRMA, we still do not know why this is so.

Recently, Nichols reformulated its hGH diagnostics reagents with standards prepared in human serum. We noted additional analytical differences between the two monoclonal IRMAs studied. The H-IRMA is highly selective for the 22-kDa form of hGH. In the serum of normal individuals about 70% of the circulating form is 22-kDa hGH, the remainder being in the 20-kDa and other forms (9). The reformulated N-IRMA demonstrated a slope of 1.3 when compared with the H-IRMA, which may be a result of the inability of the H-IRMA to detect 20-kDa hGH. There have been no documented cases of atypical 20-kDa/22-kDa ratios in human sera, so the inability to quantify 20-kDa hGH may be clinically insignificant (9). Nevertheless, the bias between the assays further underscores the necessity to determine an assay-specific reference interval. Variability in hGH results from a variety of immunoassays has recently been reported by Celniker et al. (5).

Horse serum-based quality-control material gave variable estimates of hGH concentrations. Manufactured quality-control material showed variable comparisons between assays, whereas pooled human serum quality-control material demonstrated the same discordance as patients' sera. This suggests that the somatotropin in manufactured quality-control material has immunoreactive properties differing from those of normal human serum, and may be unsuitable for the evaluation of the performance of commercial reagent kits for quantifying hGH. Alternatively, or additionally, matrix differences in commercial hGH quality-control preparations may also account for kit-to-kit disagreement, which may compromise their utility. Manufacturer-published ranges (Ciba Corning package insert, used with permission) for quality-control material can vary as much as 10-fold when commercially available reagents are used to quantify hGH: Hybritech-Tandem R 1.4 ± 0.5 μg/L; Serono Diagnostics 3.2 ± 0.9 μg/L; Radioassay Systems 6.1 ± 1.5 μg/L; Pharmacia 10.3 ± 2.6 μg/L; and Nichols Institute Diagnostics 3.4 ± 0.9 μg/L.

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Table 1. hGH Quantification in Manufactured (Ciba Corning) vs In-House Pooled Serum Quality-Control Material

<table>
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<th>NIH RIA</th>
<th>Hybritech IRMA</th>
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n = 30 each.

References