Somatotropin as Measured by a Two-Site Time-Resolved Immunofluorometric Assay

Christian Strasburger, 1,4 Geoff Barnard, 1 Luca Toldo, 1 Betys Zarmi, 1 Zvi Zadik, 2 Avinoam Kowarski, 3 and Fortune Kohen 1,4

To date, many of the current criteria for diagnosis of somatotropin (growth hormone, GH) deficiency have been based upon measurement of this hormone by competitive radioimmunoassay (RIA) with use of polyclonal antibodies. In recent years, however, the development of hybridoma technology has led to the generation of various monoclonal antibodies (Mabs) to GH with different affinities and epitope specificities. Subsequently, these reagents have been used in the development of noncompetitive two-site immunometric assays (e.g., immunoradiometric assay; IRMA). In general, the values obtained for serum GH by IRMA have been lower than those obtained by RIA, because of the epitope-specificity profile of the Mabs in the IRMA. Attempting to obtain GH values numerically similar to those by RIA, we used a combination of Mabs to GH in developing and evaluating a two-site time-resolved immunofluorometric assay (TR-FIA) based on the streptavidin–biotin interaction. Fluorescence is proportional to concentration of analyte and is linearly related to concentration over the range 0.3 to 40 µg/L. The assay was satisfactory with respect to sensitivity, accuracy, and precision (CV <10% over the entire working range). In addition, the concentration of GH was determined by the IRMA and a competitive RIA in serum obtained from GH deficient and acromegalic patients. The pairing of antibodies in the IRMA gave numerical values that agreed well with those by RIA (r = 0.97; n = 100).

Additional Keyphrases: human growth hormone · reference interval · monoclonal antibodies · immunometric assay · acromegaly · heritable disorders · dwarfism · gigantism

Several clinical conditions require accurate estimation of circulating somatotropin (growth hormone, GH). 1 For example, hypopituitarism or isolated GH deficiency, two of the several causes for small stature or dwarfism in children, are characterised by concentrations of GH below 7 µg/L in serum after different pharmacological stimulation tests (2). Occasionally, a patient may be considered to have partial GH deficiency when the highest concentrations of circulating GH after stimulation are between 7 and 10 µg/L (3). Alternatively, acromegaly, which results from the presence of a benign acidophilic pituitary tumor, may be characterised by the existence of a significantly increased GH concentration in plasma (2). In addition, gigantism is associated with continuously high GH concentrations before puberty, and chronic renal failure may result in increased GH in serum. In contrast, obesity is frequently associated with diminished secretion of GH (3).

Methods based on the principles of competitive radioimmunoassay (RIA) and involving polyclonal antibodies have been developed and applied routinely for measurement of GH in serum (4) after pharmacological stimulation tests for an assessment of GH pituitary reserve. The abundant data resulting from these procedures have proved to be clinically useful in assessing hypothalamo-pituitary function, identifying pathological conditions, and monitoring treatment.

More recently, more sensitive, precise, and potentially more specific two-site immunometric assays have been introduced (e.g., immunoradiometric assay; IRMA) for measurement of GH (5). The routine application of "sandwich"-type assays for measurement of peptide hormones, however, has raised some unexpected problems. For example, several two-site assays have been shown to be negatively biased when results were compared with the corresponding values obtained from conventional competitive immunooassays (i.e., RIA). In particular, Reiter et al. (6) compared four different assays for GH and demonstrated numerical differences in the estimates of GH obtained, although there was generally good correlation between results by the methods. Moreover, they reported that the most significant negative bias (60–70%) was obtained when results of the IRMA were compared with those by an RIA. This finding has been confirmed by other workers, both for measurement of GH (7, 8) and of other peptide hormones (9). To date, the diagnosis of GH deficiency has generally been based upon the particular numerical results (<10 µg/L) obtained by competitive RIA with use of polyclonal antibodies. Consequently, the introduction of these noncompetitive procedures has led to some difficulties in clinical interpretation, because the alternative two-site assays may have very different reference intervals.

The application of time-resolved fluorescence to immunooassay has enabled development of a wide range of very sensitive techniques for measuring peptide hormones and haptenas (10). Here we describe and evaluate a time-resolved immunofluorometric assay (TR-FIA) for measurement of GH, mediated by the streptavidin–biotin interaction. The method was assessed for specificity, sensitivity, accuracy, and precision. In addition, the concentrations of GH in serum obtained from patients with GH deficiency or acromegaly were determined by the IRMA and compared with those obtained from conventional competitive RIA involving an iodinated tracer.

Materials and Methods

Materials

Reagents. Tween 20 and Tween 40 surfactants, bovine serum albumin (BSA; "RIA" grade), human placental lactogen (hPL), Pristane (2,6,10,14-tetramethylpentadecane), polyethylene glycol (M, 7000–8000), bovine γ-globulin, incomplete and complete Freund's adjuvant, and Iodogen

1 Department of Hormone Research, The Weizmann Institute of Science, Rehovot, Israel.
2 Pediatric Endocrine Unit, Kaplan Hospital, Rehovot, Israel.
3 Department of Pediatric Endocrinology, University of Maryland, Baltimore, MD.
4 Present address: Klinik für Innere Medizin, Medizinische Universitätsklinik, Lubeck, F.R.G.
5 Address correspondence to this author.
6 Nonstandard abbreviations: GH, growth hormone; IRMA, immunoradiometric assay; IRMA, immunofluorometric assay; hPL, human placental lactogen; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Mab, monoclonal antibodies; and hPRL, human prolactin.

Received November 14, 1988; accepted March 1, 1989.

CLINICAL CHEMISTRY, Vol. 35, No. 6, 1989 913
(1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) were purchased from Sigma Chemical Co., St. Louis, MO. Biotin-
ε-aminocaproic acid-γ-butyric acid, and N-hydroxysuccinimide were from Bio-Makor, Rehovot, Israel. Sepharose–
Protein A and Sephadex G-25 PD-10 columns were from Pharmacia, Uppsala, Sweden. Anti-mouse immunoglobulin
ulins for the Ouchterlony immunodiffusion technique were from Sero tec, Oxford, U.K. Streptavidin labeled with
EDTA-chelated europium (13 mol of europium per mole of streptavidin) and enhancement solution (see below) were
donated by T. Lövgren and I. Hemilla (Wallac Oy, Turku, Finland). Carrier-free Na125I was obtained from New Eng
land Nuclear, Boston, MA. NSO mouse myeloma cells were provided by C. Milstein, Cambridge, U.K.

Reference preparations. GH and hPRL from pituitary extracts were provided by Nils Norman, Oslo, Norway. GH
reference preparation (AFP703B, GH-RP-1, NIAMDD-NIH) and the 20-kDa variant of GH were from S. Raitt,
National Hormone and Pituitary Program, NIH, Bethesda, MD. The preparation and characterization of Met14GH have been described (11). The preparation and characterization of Met Leu6GH will be reported elsewhere. These
materials were donated by T. Vogel, Biotechnology General, Rehovot, Israel.

Patients, and sample collection and storage. Heparinized
blood samples were obtained by a continuous blood-with
drawal method (12) from five subjects, including (a) a
normal child; (b) two short children who had a normal
response to two standard GH pharmacological stimulation
tests; (c) one patient whose somatotropin deficiency was
demonstrated by an abnormal GH response (<5 μU/L) to
two pharmacological stimulation tests; and (d) one acromeg
atic patient. In addition, 15 additional samples were
obtained from three acromegalic patients. The plasma was
separated by centrifugation and stored at −20 °C before
measurement as previously described (12).

Procedures

Production of monoclonal antibodies to GH. Two-month-
old female CB6F1 and CD4F1 mice (six mice per strain)
were immunized with recombinant GH in complete
Freund’s adjuvant (10 μg per mouse) at monthly intervals
by intradermal and subcutaneous injections. After six
months of immunization, the mouse of either strain show-
ing the highest serum-titer of anti-GH antibodies was
sacrificed, and the immune spleen-cells were fused in the
presence of polyethylene glycol by the hybridoma tech
ique of Köhler and Milstein (13). The cells were grown in
medium containing 150 mL of horse serum per liter accord
ing to a procedure previously described (14). The culture
supernates of the growing hybrid cells were screened for
anti-GH activity after 10 to 12 days of culture, with use of
a liquid-phase RIA procedure that can briefly be summa
rized as follows. Incubate 100 μL of culture supernate
overnight, or for 2 h at 37 °C, with 100 μL of 125I-labeled
GH [prepared by the iodogen method (15), approximately
40 000 counts/min per 100 μL] in 50 mmol/L phosphate-
buffered isotonic saline (PBS), pH 7.2, containing 5 g of
BSA and 1 g of sodium azide per liter. Precipitate the
immune complexes at 4 °C by adding 1 mL of cold polyeth
ylene glycol (200 g/L) in saline. Centrifuge at 4000 × g for
15 min, aspirate and discard the supernates, and count the
radioactivity of the pellets for 1 min in a gamma scintilla
tion counter.

The hybrid cells that were positive by RIA were cultured,
washed the strips three times with wash solution. Cover the strips and store them dry at 4 °C until use.

Two-site time-resolved immunofluorometric assay. Prepare GH standards in concentrations ranging from 0 to 40 µg/L in assay buffer [Tris HCl buffer, 50 mmol/L, pH 7.75], prepared by dissolving 6 g of Tris in 1 L of doubly distilled water that contains 5 g of BSA, 0.5 g of bovine γ-globulin, 20 µmol of diethylenetriaminepentaacetic acid, 500 mg of Tween 20, 0.9 g of sodium chloride, 0.5 g of sodium azide, and HCl sufficient to adjust the pH to 7.75. Add 50 µL of standard or serum sample to the coated microtiter wells, in duplicate. Then add 50 µL of horse serum or buffer to the wells containing the standards or the samples, respectively, followed by 100 µL of assay buffer containing biotinylated monoclonal antibodies to GH (100 ng; clone 7). After an overnight incubation at 4 °C, aspirate the reaction mixture and wash the strips three times with wash solution (prepared by dissolving 0.6 g of Tris in 1 L of doubly distilled water containing 50 mg of Tween 20, 0.9 g of sodium chloride, 0.5 g of sodium azide, and HCl sufficient to adjust the pH to 7.75).

Add 200 µL of assay buffer containing 10 ng of europium-labeled streptavidin to the microtiter wells. After a further incubation at room temperature for 1 h in an automatic plate shaker, aspirate the reaction mixture and wash the strips six times with wash solution. Then add 200 µL of enhancement solution (1% Triton X-100, 6.8 mmol of potassium hydrogen phthalate, 100 mmol of acetic acid, 50 µmol of tri-n-octylphosphine oxide, and 15 µmol of 2-naphtoylethyleneacetonate in 1 L of doubly distilled water) and agitate the strips for the shaker on 10 min. Then measure the fluorescence (we used an Arcus time-resolved fluorometer; Wallac Oy, Turku, Finland).

Radioimmunoassay. Serum GH was determined by a double-antibody method (4) with use of an iodinated antigen. The CV, both within- and between-batch, was <15% for values >5 µg/L and <5% for values between 0 and 2 µg/L. The sensitivity, defined as the least amount of GH that could be distinguished from zero (mean ± 2 SD), ranged from 0.3 to 0.8 µg/L between assays.

Calculation of IFMA results. The unknown values were derived from the calibration curves (signal vs concentration of GH, µg/L).

Results

Calibration curve and precision profile. A typical calibration curve and precision profile obtained for measurement of GH by the IFMA are shown in Figure 1. The CV was <10% across the entire working range of the assay. The same curve was obtained in 10 consecutive assays.

Specificity. The specificity of the IFMA was investigated by measuring two concentrations (1 and 10 µg/L) of structurally related compounds [22-kDa GH, 20-kDa GH, Met Leu4GH, Met4GH, hPL, and human prolactin (hPRL)]. The cross-reaction with each analyte was calculated as follows: cross reaction, % = (observed value/expected value) × 100. The results are shown in Table 1.

Precision. This was evaluated in terms of intra- and interassay CVs. The intra-assay CV was estimated by analyzing replicate samples in duplicate from three pooled sera within a single assay. The interassay CV was evaluated by measuring GH in three pooled specimens of plasma used for internal quality control during a three-month period. The results are shown in Table 2.

Sensitivity. The mean value of GH that could be distinguished from zero (mean ± 2 SD), calculated from five consecutive calibration curves prepared in duplicate, was 0.1 ng/mL, or 0.2 milli-int. units/L.

Accuracy. We investigated the accuracy of the IFMA by adding GH (range: 0.156 to 40 µg/L) to a plasma pool and assaying it. We could account for 97.2 (SD 8.2)% of the added GH.

Correlation with RIA. The concentration of serum GH was measured by the IFMA and a competitive RIA (4) in samples collected from patients with different clinical conditions. These included: (a) children with an abnormal GH response to pharmacological GH stimulation, studied by use of the continuous blood-withdrawal method (13); (b) children with a normal GH response to pharmacological GH stimulation; and (c) acromegalics (24-h spontaneous secretion studies) with GH concentration that could not be suppressed by an oral glucose-tolerance test and somatomedin C (IGF-1) concentrations of 4.5 to 6.0 kilo-int. units/L. The results are shown in Table 3.

Discussion

The introduction of competitive immunoassays in the early 1960s facilitated the development and wide application of numerous sensitive and specific techniques for measurement of haptens and analytes of high molecular mass. Consequently, during the last 25 years, a great deal of information has accumulated with respect to the clinical utility of these procedures. For example, normal and pathological conditions have been characterized by particular concentrations of circulating hormones as determined by RIA. In addition, effective clinical management has come to depend on the provision of consistent data from the laboratory. In particular, assessments of GH-deficiency syndromes have been classified according to numerical criteria obtained from competitive RIAs in which monoclonal anti-
bodies are used (4). For example, traditional tests of GH sufficiency have relied upon an acute increase in circulating GH in response to pharmacological stimulating agents such as insulin, arginine, L-dopa, or clonidine (3). Classically, normal responses of GH to these stimulation tests have provoked a circulating GH concentration >10 μg/L (1).

The introduction of two-site immunometric assays involving monoclonal antibodies has revolutionized measurement of protein hormones in biological fluids. The advantages of these non-competitive procedures have been well documented (19) and include increased sensitivity, wider working range, and better precision. In theory, the use of two antibodies should improve overall assay specificity by the recognition of two antigenic epitopes rather than one. Nevertheless, certain problems have been identified with the use of these procedures that were not anticipated.

Competitive assays involve the use of one antibody that will recognize one "face" or epitope of the analyte. Consequently, this type of assay (e.g., RIA) possibly will measure some alternative forms of the analyte that may be present in biological fluid, including (a) native forms, (b) fragments, (c) variants, and (d) protein-bound complexes. Two-site assays may or may not recognize these alternative forms. If they do not, non-random negative bias will result. Consequently, what pair of antibodies is used becomes of critical importance in the development of a non-competitive assay that will give numerical results similar to those obtained by a conventional RIA.

Measurement of GH is a typical case in point. For example, GH is known to circulate in both free and protein-bound form. For example, Leung et al. (20) demonstrated the structural identity of rabbit GH binding protein in serum with the extracellular, hormone-binding domain of the membrane-bound GH receptor. More recently, several groups have identified and characterized specific binding proteins for GH in normal human serum (21-23). Moreover, Daughaday and Trivedi (24) demonstrated the absence of GH binding protein in patients with somatotropin receptor deficiency (Laron dwarfism).

In addition, various structurally modified forms of GH have been isolated from the pituitary gland. The predominant form is a single peptide chain of 191 amino acids having a molecular mass of about 22 kDa. About 10% of the total GH in the pituitary, however, is a 20-kDa variant that lacks residues 32-46 of the amino acid sequence (25). Moreover, both biological turnover and long-term storage results in GH fragments being present in serum samples.

All of these factors may affect the performance of a non-competitive assay much more profoundly than they will affect a conventional competitive RIA. Consequently, choosing different combinations of Mabs for capture and labeled reagent will alter the ability of the immunometric assay to recognize and measure alternative biological forms of the analyte. The more "specific" the assay for a particular form of the analyte, the more negative the bias may become when the values obtained are compared with those from the "reference" RIA and, more particularly, with those from bioassay. In addition, these factors are pertinent to the uncritical use of "sandwich"-type assays for measurement of analytes in alternative types of specimens such as urine or in serum or plasma that has been frozen and thawed several times.

All of these factors are relevant to measurement of other analytes by two-site immunometric assays (9). This has resulted with a reassessment of the value of the "sandwich"-type assay, and in some cases has led to a return to use of the older reference RIAs. This is unfortunate, because the advantages of the non-competitive assays are significant. In this report, we have described the development and performance of an IFMA for measurement of the hormone in serum. The Mab combination selected (capture Mab: clone 2; labeled Mab; clone 7) gave numerical results very similar to those obtained by the comparison RIA. It may be speculated that the two epitopes recognized by these antibodies are available for binding in the alternative forms of the circulating analyte. In addition, the IFMA is satisfactory in terms of sensitivity, working range, precision, accuracy, and parallelism.

In terms of specificity, the simultaneous IFMA described here has a significant cross-reaction (mean 25.7%) with hPL (see Table 1). This makes the assay unsuitable for use.
in monitoring pregnant patients. For most of our clinical purposes (e.g., the identification of GH deficiency and acromegaly), this cross-reaction is of no consequence. Nevertheless, if the assay is performed in a sequential manner (i.e., two steps), the cross-reaction with HPL is negligible (<0.1%) and the assay is therefore suitable for use in measurement of GH in pregnant patients.

Consequently, in terms of clinical utility, the method is satisfactory. It is common knowledge that GH is secreted episodically during the 24-h period. Peaks tend to be higher towards puberty and decrease in amplitude thereafter so that in old age the concentrations of GH are very low. The occurrence of six to eight peaks during the 24-h cycle, with barely detectable concentrations in between, will cause a large standard deviation about a mean value (see Table 3). The range of values for mean 24-h GH have been published previously (26), as have the mean values in relation to age (12). Clearly, the mean GH values that are normal in adolescence are indicative of acromegaly in old people, and values that are normal in old age, when determined in a child, may be indicative of pituitary failure.

Our results suggest that, in many cases, careful selection of the appropriate Mabs can obviate most of the potential difficulties and can facilitate the development of more sensitive, precise, and yet still clinically useful diagnostic procedures. Currently, we are investigating the effect of choosing alternative antibody pairing on the clinical utility of GH measurements by two-site immunometric assays.

C.S. was supported by a Minerva Fellowship at the Weizmann Institute of Science. G.B. is a visiting Research Fellow in the Department of Hormone Research, The Weizmann Institute of Science, supported on the grant (BR-13-001) from the Institute for International Studies in Natural Family Planning, Department of Obstetrics & Gynecology, Georgetown University Medical School, Washington, DC (USAID co-operative agreement no. DPE-3040-A-005964-00). We thank Dr. Timo Lövgren and Wallace Ot, Turku, Finland, for generously providing equipment and reagents, and Dr. S. Raiti (the National Hormone and Pituitary Program, NIH, Bethesda, MD) for 20-kDa GH and reagents for the RIA.

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