

## Sensitive Time-Resolved Fluorescence Immunoassay of Somatotropin in Serum

Ileana Kahan,<sup>1</sup> Anastasia Papanastasiou-Diamandi,<sup>1</sup> Graham Ellis,<sup>2,3</sup> Sinikka K. Makela,<sup>2</sup> JoAnne McLaurin,<sup>2,3</sup> Mario D'Costa,<sup>3,4</sup> and Eleftherios P. Diamandis<sup>1,3,5</sup>

We describe a new "sandwich"-type non-isotopic immunoassay for human somatotropin (GH, growth hormone) in serum. In the assay, GH is captured by a monoclonal antibody immobilized in a white microtiter well and simultaneously reacted with a second biotinylated monoclonal antibody. The degree of binding of biotinylated antibody, which increases with increasing amount of GH in the sample, is quantified by adding streptavidin labeled with the europium chelate of 4,7-bis(chlorosulfofophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid. The fluorescent complex on the solid phase is then measured by excitation at 337.1 nm (nitrogen laser) and monitoring the emission at 615 nm in a gated fluorometer/analyzer. The proposed procedure has short incubation times (<4 h protocol), uses only 25  $\mu$ L of serum per microtiter well, and gives precise and accurate results. The method was clinically evaluated with samples obtained from pediatric patients undergoing investigation for growth abnormalities and from a patient with acromegaly.

**Additional Keyphrases:** acromegaly · growth hormone deficiency · europium chelates · peptide hormones · pediatric chemistry

Human somatotropin (GH, growth hormone), a single-chain 191-residue polypeptide, is produced by the somatotrope cells of the anterior pituitary.<sup>6</sup> The main function of GH is to stimulate the hepatic production of a family of peptides, called somatomedins, which mediate much of its action on bone and cartilage and act like GH in other tissues (1, 2). Growth in children depends on the regularity and amplitude of pulses of GH secreted by the pituitary.

Measurement of GH in serum is important in the investigation of growth abnormalities in children (3, 4) and for the diagnosis of acromegaly in adults (5). Because GH is secreted in pulsatile fashion, it is usually measured in serum of patients with possible deficiency after stimulation by one or more of the following: exercise, insulin-induced hypoglycemia, infusion with L-dopa/propranolol or arginine; or it is measured at frequent intervals during sleep (3, 4, 6). Measurement of GH in urine (7) avoids the need

for blood sampling, but is unreliable in children with renal disease (8). Suppression testing for diagnosing acromegaly or cerebral gigantism is usually done by glucose challenge (5).

In early sensitive double-antibody radioimmunoassays for GH quantification in serum, polyclonal antibodies were used (9). Although such methods are still in use, more sensitive and specific assays based on the immunometric principle are becoming more widely adopted and are more precise (10-12). Monoclonal antibodies are now being used in many two-site "sandwich type" configurations (10, 11, 13). The development of nonisotopic techniques has eliminated the hazards associated with the use of radiolabeled tracers, and allows use of reagents with longer shelf-lives. The outcome has been the introduction of a new generation of nonisotopic immunoassays for GH in serum or urine, with detection systems that incorporate enzyme, luminescent, or fluorescent probes as labels (14, 15). A particle-counting immunoassay of human GH has also been described (16).

Fluorescent europium complexes are useful as alternative labels to radioisotopic compounds in immunoassays. Europium has a long fluorescence decay time, a sharp emission band, and a large Stokes shift. Fluorescence from europium complexes can be detected with very high sensitivity and low background signal by time-resolved fluorometry (17, 18).

Here we present a fluorescence immunoassay for GH in serum. The assay, based on the "sandwich" principle, is performed in microtiter wells coated with a monoclonal antibody to GH. Biotin-labeled monoclonal antibody is used for detection. The degree of binding of the biotinylated antibody to captured GH molecules is determined by a bridge reaction with a europium-saturated chelate, 4,7-bis(chlorosulfofophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA), covalently attached to streptavidin. The fluorescence of the final complex formed (antibody<sub>1</sub>-GH-antibody<sub>2</sub>-biotin-streptavidin-BCPDA-Eu<sup>3+</sup>) is then quantified in the dried solid phase by pulsed fluorescence measurements with a gated fluorometer/analyzer.

To evaluate the assay clinically, we used it for testing GH in 69 patients who were being evaluated for possible GH deficiency and in one patient with acromegaly.

### Subjects and Methods

#### Instrumentation

For solid-phase time-resolved fluorometric measurements, we used the "CyberFluor 615 Immunoanalyzer" (CyberFluor Inc., Toronto, M5T 1X4, Canada). This instrument has automatic data reduction. For radioactivity counting we used a Model 1275 "Minigamma" counter (LKB Wallac, Turku, Finland).

#### Reagents

**Chemicals.** Bovine serum albumin and streptavidin were from Sigma Chemical Co., St. Louis, MO 63178; europium(III) chloride hexahydrate was from Aldrich Chemical

<sup>1</sup> CyberFluor Inc., 179 John St., Toronto, M5T 1X4, Canada.

<sup>2</sup> Department of Biochemistry, The Hospital for Sick Children, 555 University Ave., Toronto, M5G 1X8, Canada.

<sup>3</sup> Department of Clinical Biochemistry, University of Toronto, 100 College St., Toronto, M5G 1L5, Canada.

<sup>4</sup> Department of Clinical Biochemistry, Mount Sinai Hospital, 600 University Ave., Toronto, M5G 1X5, Canada.

<sup>5</sup> Address correspondence to this author at CyberFluor Inc.

<sup>6</sup> Nonstandard abbreviations: GH, growth hormone; BCPDA, 4,7-bis(chlorosulfofophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid; and FIA, fluorescence immunoassay.

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Co., Milwaukee, WI 53233; sulfosuccinimidyl 6-(biotinamido) hexanoate ("NHS-LC-Biotin") was from Pierce Chemical Co., Rockford, IL 61105; white opaque 12-well microtiter strips ("Microfluor") were products of Dynatech Laboratories Inc., Alexandria, VA 22314.

**Buffers.** The coating buffer was a 50 mmol/L carbonate solution, pH 9.6. The blocking buffer was 0.1 mol/L carbonate solution, pH 8.3, containing 10 g of BSA and 0.5 g of sodium azide per liter. The assay buffer was a 50 mmol/L Tris solution, pH 7.80, containing 29.8 g of KCl, 10 g of bovine serum albumin, and 0.5 g of sodium azide per liter. The streptavidin-europium buffer was a 50 mmol/L Tris solution, pH 7.20, containing 9 g of NaCl, 40 g of bovine serum albumin, and 0.5 g of sodium azide per liter. The wash solution was a 9 g/L NaCl solution containing 0.5 mL of polyoxyethylene sorbitan monolaurate ("Tween 20") per liter.

**GH standards.** A reference preparation of human GH—NIDDK-hGH-RP-1 (AFP 4793B)—was obtained from the National Institutes of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD. The preparation was reconstituted as directed and diluted in normal equine serum (Gibco Laboratories, Grand Island, NY 14072) to give the desired standard concentrations. For commercial production, CyberFluor uses GH standard preparation from Scripps Laboratories, San Diego, CA 92131, diluted in equine serum and calibrated against the NIDDK standard; standards prepared in GH-free human serum (cat. no. 3SH100; Scantibodies Laboratory, Santee, CA 92071) currently are under evaluation.

**Monoclonal antibodies.** Two mouse antibodies raised against human GH were used. These antibodies, immunoglobulin fractions purified from the ascites fluid by ion-exchange chromatography, are available through CyberFluor Inc.

## Samples and Patients

Human serum samples were stored at  $-20^{\circ}\text{C}$  for no longer than two months until assay.

**Control subjects.** Samples were taken from 59 apparently healthy nonfasting individuals, ages 20–45 years (28 men and 31 women).

**Patients under investigation for possible GH deficiency.** This group of 69 patients was evaluated for possible GH deficiency. Residual samples left over from routine testing were used for the method evaluation.

The case records were reviewed and the patients were grouped as follows (number of patients in parentheses): growth hormone deficiency (7); constitutional short stature, with or without delayed puberty (24);  $\beta$ -thalassemia (12); Turner's syndrome (9); cancer with or without radiation treatment (5); other chronic diseases (7: three renal, two gastrointestinal, and two anemia); and miscellaneous (5: one Down's syndrome; one mental retardation; one ketotic hypoglycemia; one "MELAS" syndrome, with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; and one neurofibromatosis).

The patients all had multiple samples taken during sleep, after exercise, or during stimulatory test procedures with infusion of arginine, insulin, or L-dopa/propranolol.

Another group of 29 patients—whose diagnoses were unknown to us, whose stimulatory tests were incomplete, or from whom single or multiple samples had been drawn either during spontaneous hypoglycemia or without any specific stimulus—provided 130 additional samples that we

used in the correlation studies.

**Acromegalic patient.** A 69-year-old man had been diagnosed as acromegalic 20 years previously, based on clinical, radiological, and laboratory findings. He had refused surgical treatment and was admitted on this occasion for complications of his acromegaly (diabetes and spinal stenosis). The presence of a right pituitary adenoma was confirmed by computer tomographic scan and magnetic resonance imaging. As part of the patient's re-evaluation, he underwent a thyroliberin (thyrotropin-releasing hormone) stimulation test, an oral glucose tolerance test, and a gonadoliberin (gonadotropin-releasing hormone) stimulation test, all performed according to standard procedures.

**Control sera.** Lyphocheck immunoassay control serum (human), Levels I, II, and III, were from Bio-Rad Clinical Division, Richmond, CA 94801.

## Procedures

**Preparative procedures.** The biotinylation procedure was as previously described (19). After dialysis, we tested the biotinylated antibody at various dilutions to determine the optimal concentration for the assay. Routinely, the stock preparation, stored at  $4^{\circ}\text{C}$ , is diluted in the assay buffer just before use to give a working biotinylated antibody solution of  $2\ \mu\text{g}/\text{mL}$ . A streptavidin-bovine thyroglobulin-BCPDA conjugate was synthesized, diluted in a  $\text{Eu}^{3+}$ -containing buffer, and used as described elsewhere (20, 21). Antibody-coated microtiter wells were prepared as previously reported (19), each well being coated with 500 ng of antibody in  $100\ \mu\text{L}$ .

**Immunoassay procedure.** Add, in duplicate,  $75\ \mu\text{L}$  of assay buffer;  $25\ \mu\text{L}$  of standards, controls, or patients' samples; and  $50\ \mu\text{L}$  of the biotinylated antibody working solution to the antibody-coated wells. (Add the antibody strip by strip after the serum had been added to all 12 wells in each strip, to minimize intra-plate drift.) When the plate has been processed, cover it, shake it for 4 min in an automated shaking device, and incubate it at  $37^{\circ}\text{C}$  for 3 h. Aspirate the reaction mixture and wash the wells four times with the wash solution. (We used a 12-well aspirating-washing device.) Pipet  $100\ \mu\text{L}$  of the BCPDA-streptavidin conjugate working solution into each well, incubate for 45 min at  $37^{\circ}\text{C}$ , then wash the wells four times with the wash solution and let them dry for 5 min in cool air. Measure the fluorescence on the dried solid phase. Measurement with the CyberFluor 615 Immunoanalyzer is convenient, and it also performs the data reduction, using a spline curve-fitting procedure.

**Other methods.** Samples were assayed at the Hospital for Sick Children by our in-house RIA method (22). A commercially available immunoradiometric procedure (Allegro hGH; Nichols Institute, San Juan Capistrano, CA 92675) was also used as a comparative method. (These kits incorporated the recently introduced standards in human serum instead of the previously used equine-serum-based standards.)

## Results

**Detection limit and dynamic range of the assay.** Figure 1 shows a typical dose-response curve. The assay has a working range of 0.5 to  $50\ \mu\text{g}/\text{L}$ . To study the "high-dose hook effect," we measured the response of the assay at GH concentrations as high as  $1000\ \mu\text{g}/\text{L}$ . The fluorescence signal obtained exceeded that of the  $50\ \mu\text{g}/\text{L}$  standard in all cases. The detection limit of the assay, as calculated from

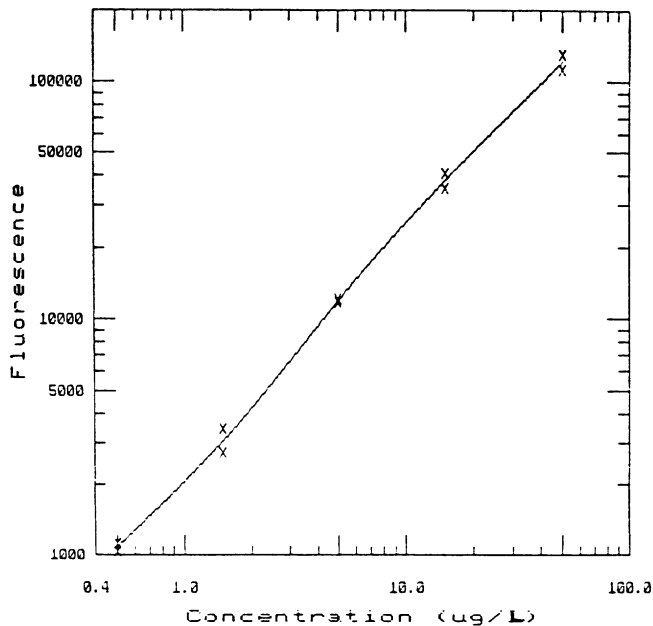


Fig. 1. Typical standard curve for GH by time-resolved fluorescence immunoassay (FIA)

the mean fluorescence plus three standard deviations of the zero standard, was  $\leq 0.1 \mu\text{g/L}$ .

**Precision.** For precision studies we used human sera and human control sera. The results are summarized in Table 1.

**Dilution linearity and analytical recovery.** To evaluate linearity, we serially diluted 10 different clinical samples with the zero standard and assayed. As shown in Table 2 for three representative samples, the concentration of GH decreased linearly with increasing dilution. The correlation between the expected and observed values was excellent in all 10 cases.

Analytical recovery experiments were performed in several ways. We prepared a stock GH preparation in horse serum,  $100 \mu\text{g/L}$ , and supplemented serum samples with either 10 or  $20 \mu\text{g}$  of GH per liter. We then calculated recoveries (mean  $\pm$  SD), using standard curves prepared with standards in either horse serum or GH-free human serum. The recoveries were  $99.6 \pm 4.2\%$  vs standards in horse serum, and  $116.8 \pm 4.9\%$  vs standards in GH-free human serum. In a similar experiment we used a  $100 \mu\text{g/L}$

Table 1. Precision of Fluorescence Immunoassay for GH

Sample	n	GH, $\mu\text{g/L}$		CV, %
		Mean	SD	
<i>Within batch</i>				
Patient 1	10	7.43	0.36	4.9
Patient 2	10	8.49	0.43	5.0
Patient 3	9	19.48	1.75	9.0
Patient 4	10	30.27	1.73	5.7
<i>Between batch</i>				
Quality-control serum A	19	1.56	0.09	5.5
Quality-control serum B	19	5.97	0.32	5.3
Quality-control serum C	19	13.31	1.35	10.2
Patient 5	16	35.00	4.11	11.7

Table 2. Linearity of Dilution of Sera with Different GH Contents ( $\mu\text{g/L}$ )

Sample		Dilution				
		None	1/2	1/4	1/8	1/16
1	Expected	—	2.41	1.21	0.60	0.30
	Observed	4.83	2.41	1.38	0.71	0.33
2	Expected	—	4.10	2.05	1.02	0.51
	Observed	8.20	3.52	1.71	0.86	0.44
3	Expected	—	7.90	3.95	1.98	0.99
	Observed	15.8	7.46	3.64	1.74	0.84

stock GH preparation in GH-free human serum rather than in horse serum. Recoveries were  $106.0 \pm 8.6\%$  with calibrators in GH-free human serum and  $85.3 \pm 4.18\%$  with calibrators in horse serum. Additionally, we compared the fluorescence values of standard curves, using standards prepared in either GH-free human serum or horse serum. We found that the horse-serum-based standards gave  $\sim 17\%$  higher fluorescence readings at GH concentrations from 1.5 to  $50 \mu\text{g/L}$ . Possible reasons for this matrix effect are discussed later.

**Cross-reactivity.** Table 3 shows the cross-reactivity of the assay, expressed as the ratio of the GH equivalent concentration resulting from the cross-reacting hormone over the concentration of the hormone that produced the cross-reactivity, expressed as a percentage. Cross-reactivity with other peptide hormones is negligible.

**Correlation with a radioimmunoassay procedure.** GH was measured by in-house RIA (22) and by the fluorescence immunoassay (FIA) method in 509 serum samples from the patients described in *Samples and Patients* (Figure 2). There was a good correlation ( $r = 0.985$ ), with intercept  $0.05 \mu\text{g/L}$  and a slope of 0.840. We attributed the slope to a matrix effect in the present assay because of the use of horse serum base for the standards (see *Discussion*).

**Correlation with an immunoradiometric procedure.** GH in 79 clinical samples was assayed by the FIA method and the Allegro commercial immunoradiometric procedure. There was generally good agreement ( $r = 0.993$ ) between results obtained by the two procedures (Figure 3), but differences in standardization, standard matrix, and (or) epitope recognition resulted in a slope of 0.740 and an intercept of  $-0.10 \mu\text{g/L}$ .

**Expected values.** Serum samples were collected at no specific time from a mixed population of 59 apparently healthy non-fasting individuals, 20–45 years old, and assayed by the proposed procedure. The distribution of values is shown in Figure 4. Most of the values were  $< 5 \mu\text{g/L}$ , as would be expected in the absence of any stimulation.

**Clinical studies.** Figure 5 summarizes the results of the stimulation and suppression testing on the acromegalic patient. The responses measured by FIA were similar to

Table 3. Cross-Reactivity Studies

Hormone	Concn. tested	Cross-reactivity, %
Somatotropin	—	100
Prolactin	$400 \mu\text{g/L}$	0.003
Follitropin	300 int. units/L	0.003
Thyrotropin	1000 int. units/L	0.001
Lutropin	250 int. units/L	Not detectable

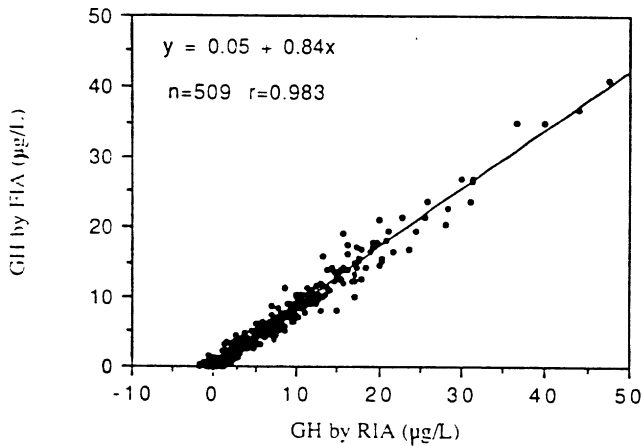


Fig. 2. Correlation between results by fluorescence immunoassay (FIA) and in-house RIA

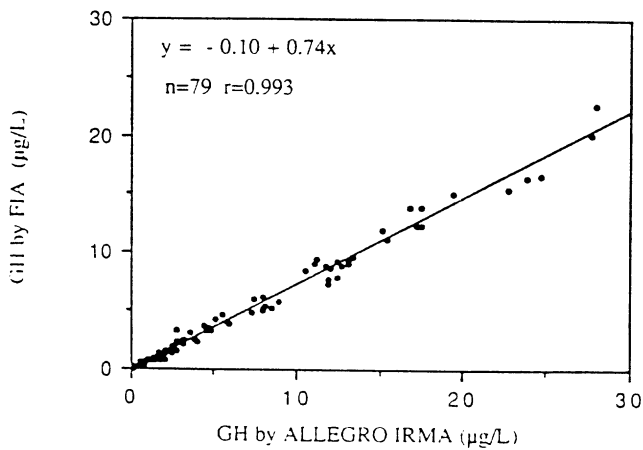


Fig. 3. Correlation between results by fluorescence immunoassay (FIA) and an immunoradiometric assay from Nichols Institute (IRMA)

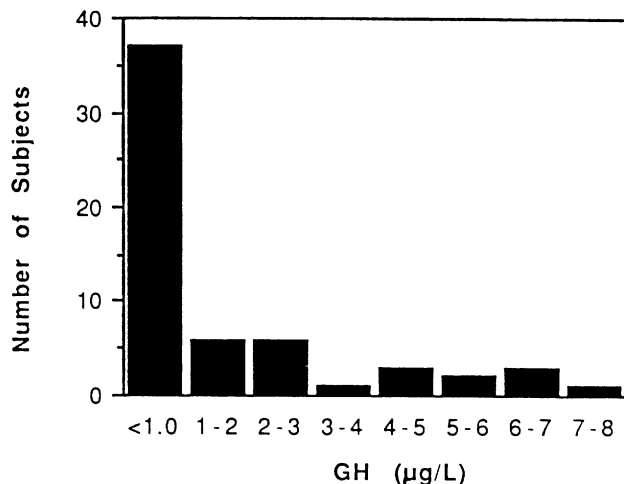


Fig. 4. Distribution of GH values in 59 nonfasting adults

those measured by an immunoradiometric assay from Pharmacia (Canada) Inc., Dorval, Quebec, Canada, H9P 1H6 (data not shown). There was partial suppression of high-baseline serum GH after a glucose challenge, followed by an increase in response to endogenously released insulin. GH was stimulated by thyroliberin and by gonadoliberein. Responsiveness to at least one of these or other stimuli is found in two-thirds of patients with acromegaly (23).

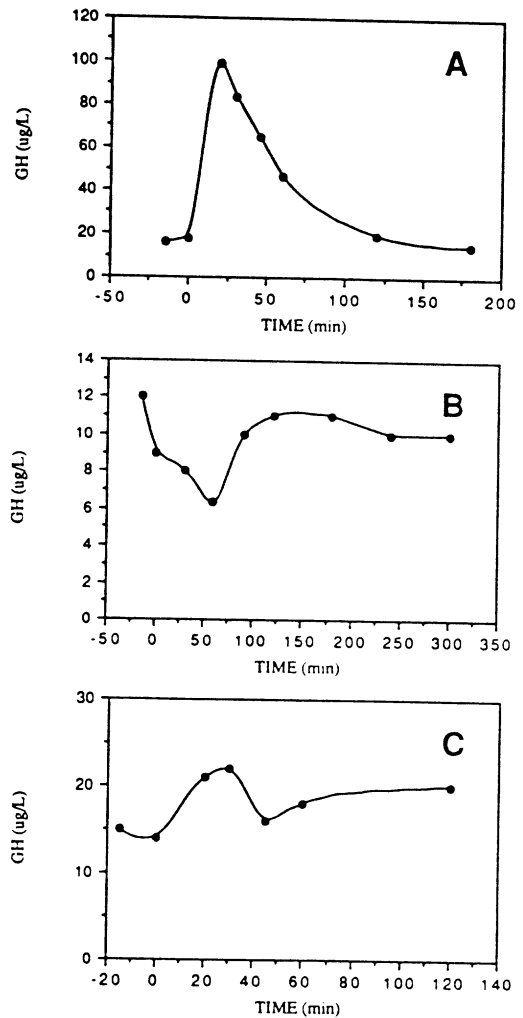


Fig. 5. Results by the present method for a patient with acromegaly: GH response to (A) thyrotropin-releasing hormone, (B) oral glucose, and (C) gonadotropin-releasing hormone

The 69 children we investigated for possible GH deficiency had at least one stimulatory test performed and many had several. The peak value is used in diagnosis, with concentrations of  $\leq 7 \mu\text{g/L}$  on several tests indicating GH deficiency in the presence of other clinical criteria (3). The peak GH observed in any stimulatory test for the individual patients is shown in Figure 6. The patients with GH deficiency had low values, and most patients in other groups (except malignancy) had values within normal limits. There was some overlap in values between the GH-deficiency and other groups, and the failure of non-

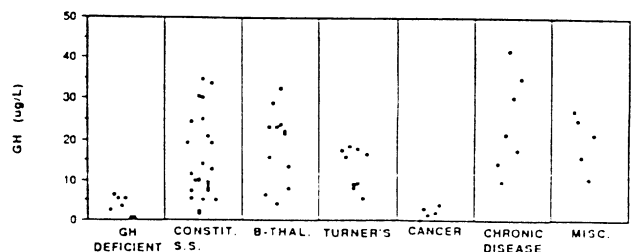


Fig. 6. Results by the present method for 69 patients investigated for possible GH deficiency  
 CONSTIT. S.S.: constitutional short stature; B-THAL.:  $\beta$ -thalassemia; TURNER'S: Turner's syndrome; MISC.: miscellaneous

GH-deficient patients to achieve an acceptable peak value may represent physiological variation in individual response to the stimulation or impaired secretion because of their disease. In such individuals, further testing or careful measurement of growth rate would be indicated. Some of the cancer patients had received chemotherapy or radiation to the head and neck, and this most probably affected their GH responses.

## Discussion

The time-resolved fluorescence immunoassay procedure described here is characterized by the following principal advantages in comparison with existing techniques. It has short incubation times, and assay of a batch of 100 samples may be completed in 5 h. It requires only 50  $\mu\text{L}$  of sample per duplicate assay, making it suitable for use in pediatrics. It is highly sensitive, with a detection limit  $\leq 0.1 \mu\text{g/L}$ , and it gives much higher fluorescence counts than those of an alternative time-resolved fluorescence procedure for GH that is potentially vulnerable to contamination with europium (15). Radioisotopes are not used and the reagents are stable for at least six months. The general performance characteristics of the assay are similar to those of existing techniques (12). The precision is satisfactory, and accuracy indices (recovery, comparisons, linearity) show good performance. The hook effect (24) does not exist for at least up to 1000  $\mu\text{g/L}$ . Cross-reactivity with other pituitary hormones is very low (Table 3). The assay appears to be clinically relevant in children under investigation for GH deficiency and in the single case of GH excess. Because different forms of GH are found in some acromegalic sera (25), patients should be studied further before concluding that the assay will be suitable for all patients with this condition.

Pituitary GH consists of several distinct molecular forms that can be separated by gel filtration chromatography (26, 27). At least three monomeric variants and several oligomers (up to a pentameric aggregate) circulate in blood (28). The major monomeric form has a relative molecular mass of 22 000 and comprises about 73% to 77% of the total monomeric GH (28). In this study, we did not try to calculate the relative immunoreactivities of monomeric and polymeric forms of GH.

Reports in the literature confirm that commercially available kits for GH in serum produce results with large discrepancies in absolute values (29–31). This observation makes interpretation of results between laboratories difficult when different kits are used (32). Possible reasons for the discrepancy are differences in the primary calibration materials, the standards matrix (29, 31), or the specificity of antibodies.

The structures of the rabbit and human GH receptors have recently been determined (33, 34). Some workers think that the extracellular domains of the receptor molecules are released from cells into the circulation and may be the source of a GH-binding protein in serum (35). Others consider the receptor and binding proteins to be different cleavage products of a single m-RNA transcript in both the rat (36) and the mouse (37). A variable fraction of the total serum GH is bound to these binding proteins [26–59%, depending on the GH isoform, with lesser proportions bound at serum GH  $> 20 \mu\text{g/L}$  (38)]. The serum GH-binding protein activity increases during childhood (39). It has yet to be determined whether these newly-described binding proteins have any possible interfering effects on GH immunoassays. However, during recovery experiments, we ob-

tained different results depending upon whether we used horse serum or GH-free serum as the matrix of both the stock and the calibration solutions. We observed higher fluorescence readings with horse serum as the matrix, suggesting that either (a) GH in human serum normally is partly bound to some serum component(s), such as protein(s), and the bound fraction is not fully available to the antibodies used, or (b) human serum has available GH-binding activity so that, when supplemented with GH, some of the hormone binds. An alternative explanation would be that a component of horse serum releases GH from a bound or aggregated form in the reference preparation and makes an additional 10–15% available for binding. The matrix effect that we observed is much less significant than that observed with a commercial immunoradiometric assay (29). That kit gave 2.7 times greater results than both a reference immunoassay and an alternative immunoradiometric assay when it incorporated standards in horse serum. It is apparent that these types of matrix effect are very dependent upon the epitopes recognized by the monoclonal antibodies.

Recent advances in site-directed mutagenesis have shown the specific amino acid residues and epitopes that are important for GH binding to its receptor (40) and to several monoclonal antibodies (41). Others have determined which parts of the GH molecule are most antigenic, without regard to bioactivity (42). Future studies may ultimately lead to the production of monoclonal antibodies directed against functional (or bioactive) epitopes on the GH molecule, similar to those produced against lutropin (43). International agreement on which epitopes are most relevant to receptor binding or bioactivity—and therefore which antibodies are preferable for immunoassays—could lead to improved standardization of immunoassays for GH. Until then, the assay described here and similar ones will continue to provide clinically useful information on patients with disorders of GH secretion.

It is likely that the number of GH tests performed in clinical laboratories will increase in future years. GH produced biosynthetically by recombinant DNA technology is becoming more readily available. It is currently being tested on various groups of short children who are not overtly GH deficient, and the debate as to who should receive GH is ongoing (4, 44). Also there is increased emphasis on GH measurement during sleep, a physiological stimulus, rather than after pharmacological infusion of arginine or insulin. Because of the variability of GH release during sleep (6), samples are usually taken at 15- to 30-min intervals during the night via an indwelling catheter, and this protocol generates six to 12 times as many samples as are taken in the course of alternative forms of testing.

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