

An in-house enzyme-linked immunoabsorbant assay for human growth hormone

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Abstract

A simple, non-isotopic in-house enzyme-linked immunoabsorbant assay (ELISA) for human growth hormone (GH) was developed. The assay involved using in-house polyclonal anti-GH adsorbed onto 96-well microtitre plates, commercially prepared mouse monoclonal anti-GH, and goat anti-mouse IgG horseradish peroxidase detection system. Results of recovery and parallelism studies ranged from 95%-106% and 98%-101% respectively, of the expected values. The detection limit of the assay was 0.008 mIU/well or the equivalent to 0.4 mIU/L of undiluted serum. Intra- and interassay coefficients of variations were 4.8%-7.9% and 6.5%-8.7% respectively. Serum GH levels measured in this assay correlated well with those measured in established in-house radioimmunoassays ($r = 0.985$, $p < 0.001$) and immunoradiometric assay from NETRIA ($r = 0.984$, $p < 0.001$).

Key words: Enzyme-linked immunoabsorbant assay, human growth hormone

INTRODUCTION

Measurement of human growth hormone (GH) levels is important in the investigation and diagnosis of GH deficiency causing growth retardation in children,^{1,3} and GH excess causing acromegaly in adults.^{4,5} The most widely used methods today are immunoassays, utilising radioisotopic labels such as 125-iodine as in radioimmunoassay (RIA) and immunoradiometric assay (IRMA).⁶⁻⁸

Although reagents for these assays are commercially available, they are expensive, and supply can be erratic in this part of the world. Prompted by these problems and our awareness that GH measurements vary in different immunoassay systems,⁹⁻¹¹ we established our own in-house RIA for GH measurements.¹² However, preparation of the radioiodinated tracer can be laborious, and its period of usage limited due to the short half-life of 125-iodine. In this paper, we describe our experience in developing a simple, non-isotopic enzyme-linked immunoabsorbant assay (ELISA) utilising our own in-house polyclonal anti-human GH, and commercially prepared, but stable and easily available mouse monoclonal anti-human GH and goat anti-mouse IgG horse-radish peroxidase conjugate.

MATERIALS AND METHODS

Polyclonal anti-GH

Polyclonal anti-GH (PAB-GH) was raised in two 3-4 month old New Zealand white rabbits by the modified methods of Vaitukaitis *et al.*,¹³ using purified human pituitary GH. For both rabbits, the best antiserum titres were obtained between weeks 12-16 after immunisation, with an affinity constant of 6.6×10^{10} L/M. A specificity study showed that the antisera cross-reacted only minimally with prolactin or human placental lactogen (<0.2% for both hormones). Neat PAB-GH aliquots were lyophilised and stored at 4°C, while the diluted aliquots were stored frozen at -40°C until used.

Commercial antibodies

Mouse monoclonal GH antibody (MAB-GH) MAB 653, clone 54/9, with specific activity 5.2×10^9 L/M) was purchased from Chemicon International Inc., California, USA, while the horse-radish peroxidase conjugates of goat anti-mouse IgG (HRP-GAM) and goat anti-rabbit IgG (HRP-GAR) were obtained from Bio-Rad Laboratories, Richmond, USA. All commercial antibodies were found to be stable for more than one year and were kept at 4°C at all times as recommended by the manufacturers.

Standard and quality control samples

The reference GH standard used was IS 80/505 obtained from the National Institute for Biological Standard and Control (NIBSC), London. Internal quality control (IQC) sera were prepared at 4 different GH levels from pooled patients' sera. The sera were aliquoted accordingly, and stored frozen at -20°C until used.

Buffers

All chemicals used were of analar grade and amounts quoted below were for the preparation of a one litre solution.

Phosphate buffered saline (PBS, pH 7.2): Na_2HPO_4 1.28g, KH_2PO_4 0.13g, NaCl 9.0g, thiomersal 0.1g. Buffer was found to be stable up to 3 months at room temperature.

Assay buffer: PBS containing 0.5% (w/v) bovine serum albumin, stable up to 1 month at 4°C .

Coating buffer (pH 9.6): Na_2CO_3 1.5g, NaHCO_3 2.94g. Stable up to 2 months at 4°C .

Blocking buffer: PBS containing 2% (w/v) BSA. Prepared when required.

Citrate-phosphate buffer (pH 5): Na_2HPO_4 7.1g, citric acid 5.25g. Stable up to 2 months at 4°C .

Substrate buffer: Citrate-phosphate buffer 25ml, *o*-phenylene diamine dihydrochloride (OPD) 10mg, H_2O_2 (30% v/v) 10ul. Prepared within 30 minutes of use.

Stopping solution: 1.25M sulphuric acid.

ELISA procedure

The 96-well flat-bottomed microtitre plate was Nunc Immunoplate Maxisorp F96 (with certificate), purchased from Nunc, Roskilde, Denmark. Samples were assayed in duplicate; all incubations were performed at ambient temperature. The assay procedure is as shown in the flow chart (Fig. 1). Wells were first coated with 100 ul PAB-GH which had been diluted 20,000 fold with coating buffer. After 4 hours, wells were washed 3 times with wash buffer, thoroughly dried, then filled with 200 ul of blocking buffer. One hour later, blocking buffer was discarded, and after tapping the plate dry onto an absorbant paper, 20 ul of standard/IQC/samples and 80 ul of assay buffer were added to each well. The plate was firmly covered with parafilm, and incubated for 20 hr on a plate shaker (rotating at 150 rpm). Following 3 cycles of washing and thorough drying, 100 ul of MAB-GH at a concentration of 200 ng/ml was dispensed into each

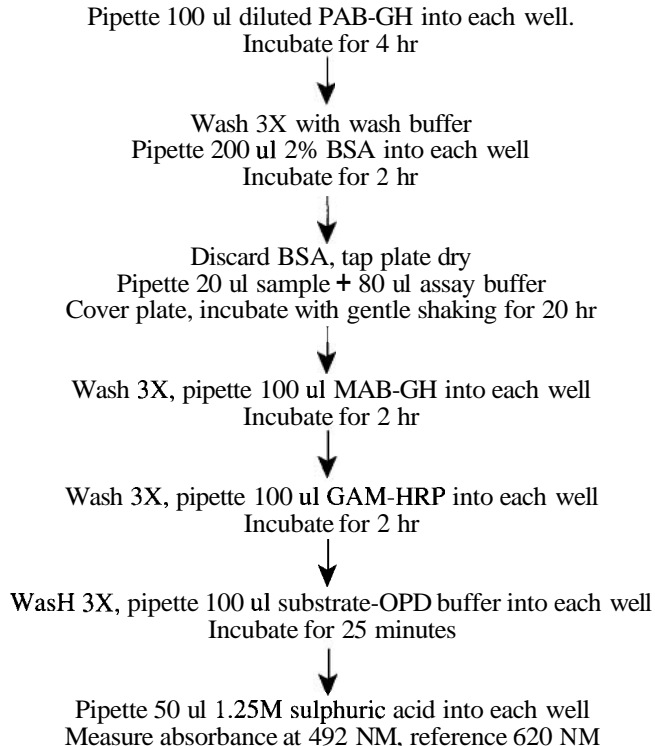


FIG. 1: Flow-chart for GH ELISA.

well. The plate was again incubated for 2 hr, then washed 3 times. A hundred μ l of freshly prepared GAM-HRP (1:6,000 dilution) was then added and the plates incubated for 2 hr. After another cycle of washing and drying, 100 μ l of freshly prepared substrate buffer was added to each well. The plate was incubated for 25 minutes in the dark, following which 50 μ l of 1.25M sulphuric acid was immediately added to terminate the chromogenic reaction. After gentle tapping to mix the contents, absorbance was read at 492 nm (with reference at 620 nm).

Assay optimisation

Working titres for PAB-GH and MAB-GH: Wells were coated with PAB-GH at dilutions of 1:1,000, 1:20,000 and 1:100,000, while MAB-GH was titrated at concentrations of 10, 20 and 40 ng/well. Detection was by using HRP-GAM conjugate at a dilution of 1:3,000. In another set of plates, wells were coated with MAB-GH at concentrations of 156, 312 and 625 ng/well, while PAB-GH was used at dilutions of 1:200,000 and 1:400,000 and HRP-GAR conjugate at 1:10,000 dilution.

Matrix effect: Matrix effect was determined by assaying, simultaneously, 2 sets of GH standard curves. One set was prepared in buffer, while the other set contained 20% of GH-free charcoal-stripped human serum.

Assay validation

Validity of the in-house ELISA was assessed as follows:

Recovery: Serum samples were assayed before and after quantitative addition of exogenous GH at concentrations of 3.1, 52 and 106.2 mIU/L.

Parallelism: Serum samples of acromegalic patients with GH levels ranging from 25.9-68.5 mIU/L were serially diluted with assay buffer, and then assayed neat and at dilutions of 1:2, 1:4 and 1:8.

Correlation study: Patients' samples were simultaneously assayed in 3 different assay systems; the in-house ELISA, the in-house RIA which had been reported and assessed in the UK-EQAS, Edinburgh ($r = 0.975$, $y = 1.02x - 0.7$, $p < 0.001$)¹¹ and the IRMA from North-East Thames Region Immunoassay Unit (NETRIA).

Statistics

All assay results were analysed using the LKB-Wallac RIA-CALC program while correlations

between assay methods were analysed by Spearman's rank test.

RESULTS

When PAB-GH was used as the coating antibody, optimal standard displacement was obtained when the antiserum was at a dilution of 1:20,000 and the concentration of MAB-GH was 20 ng/well (Figs. 2a and 2b). On the other hand, the working titre for PAB-GH could be increased to 1:200,000 and 1:400,000 when wells were first coated with a high concentration of MAB-GH (625 ng/well) (Fig. 3). However, since the latter had to be purchased commercially, we chose to use the method of coating plate with our own in-house PAB-GH which was available in sufficient amounts for our need.

Repeated assays showed that there was no significant difference in the standard displacement when GH standards was prepared only in assay buffer or in the presence of 20% GH-free human serum (that is, 20 μ l/well, similar to the amount of patient's serum used in the assay) (Fig. 4).

Results of recovery and parallelism studies are shown in Fig. 5 and Table 1 respectively. Excellent analytical recoveries (between 95% - 106%) were obtained for exogenous GH standards of concentrations ranging from 3.1 to 106.2 mIU/L. Similarly, high GH samples diluted up to 8-fold, showed very good recoveries (98% - 101%) in the assay (Table I).

Sensitivity of the assay, defined as the lowest detectable concentration of GH that is different from zero at 95% confidence limit, was 0.008 mIU/well which corresponds to 0.4 mIU/L in the undiluted samples. Assay imprecision, determined by repeated analyses of the four IQC sera, gave excellent results (Table 2). The intra-assay coefficients of variation (CVs) at GH concentrations of 3.4, 11.8, 19.1 and 55.7 mIU/L were 6.2, 4.8, 5.3 and 7.9% respectively, whilst the corresponding inter-assay CVs were 6.5, 5.9, 5.8 and 8.7% respectively.

Results of correlation analyses between GH levels measured by different immunoassay systems are shown in Figs. 6 and 7. The in-house ELISA correlated very well with our in-house RIA ($r = 0.985$, $y = 1.02x - 0.05$, $p < 0.001$, $n = 67$) (Fig. 6) and the IRMA from NETRIA ($r = 0.984$, $y = 0.89x + 0.84$, $p < 0.001$, $n = 61$) (Fig. 7).

DISCUSSION

For the last 3 decades, quantitative measurements of a number of clinically important proteins and peptide analytes such as hormones,

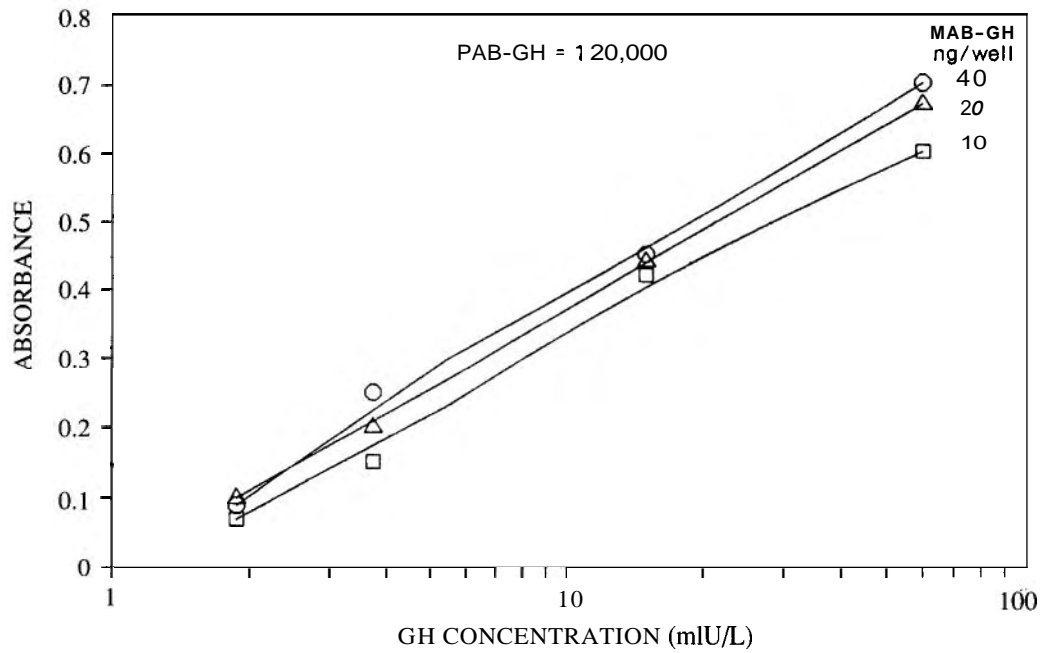
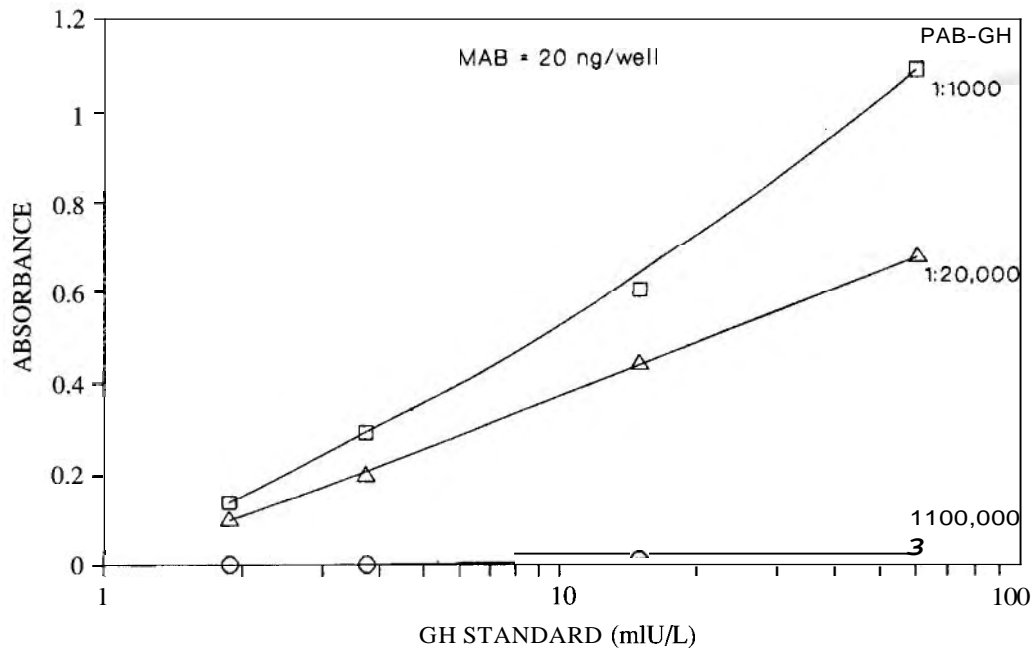


FIG. 2: Optimisation of antibody titres used in GH ELISA.
 a: Polyclonal anti-GH (PAB-GH) as coating antibody.
 b: Monoclonal anti-GH (MAB-GH) as second antibody.

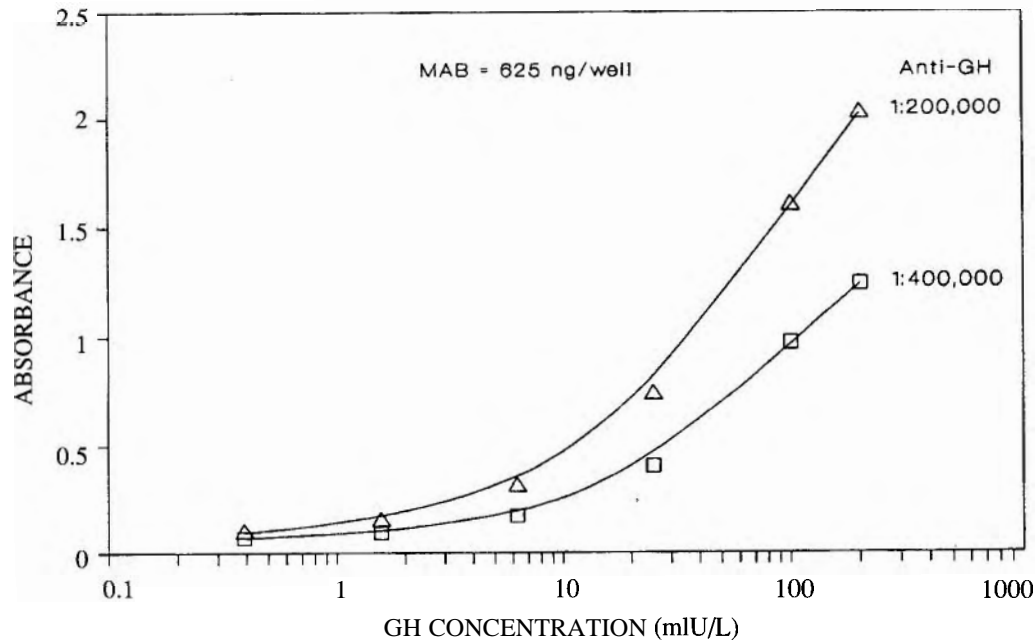
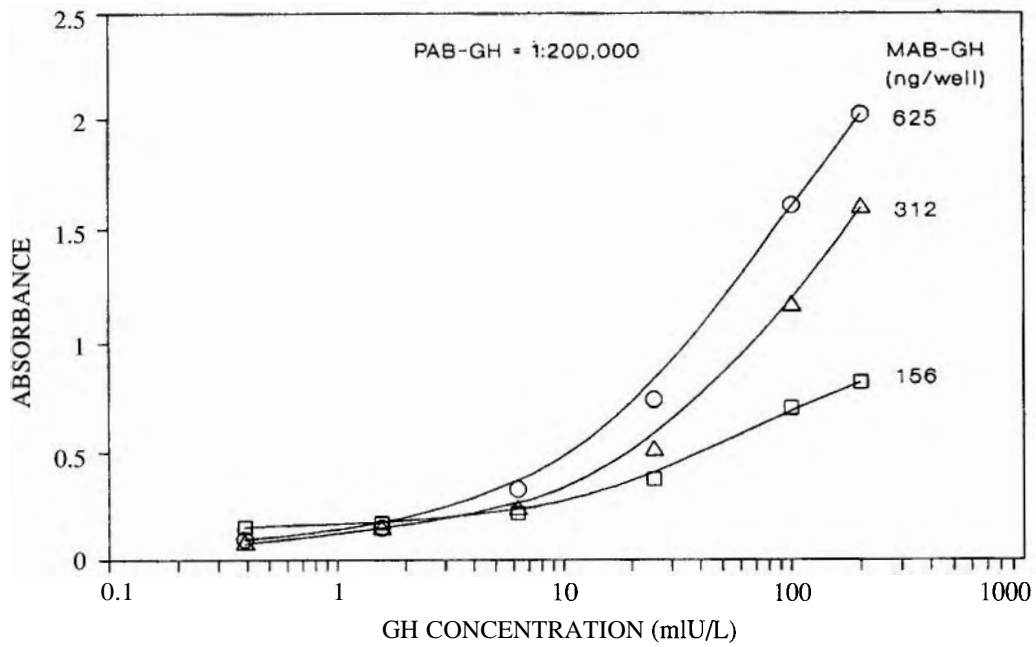


FIG. 3: Optimisation of antibody titres used in GH ELISA.
 a: MAB-GH as coating antibody.
 b: PAB-GH as second antibody.

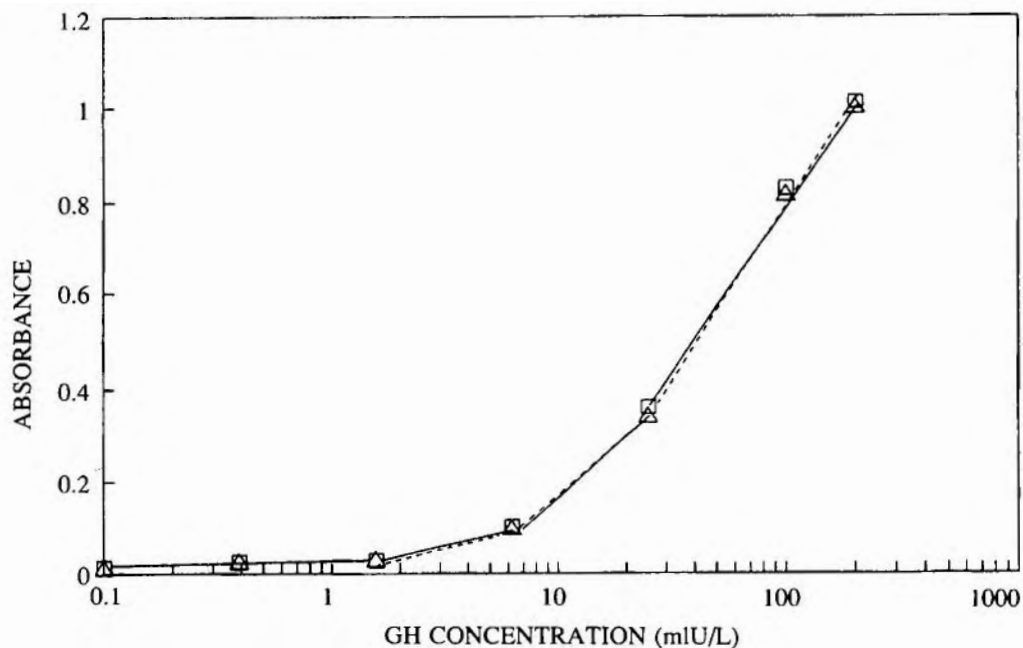


FIG. 4: Effect of matrix on GH standard curve, i.e., when standards are prepared only in assay buffer (□) and in the presence of 20%GH-free human serum (△).

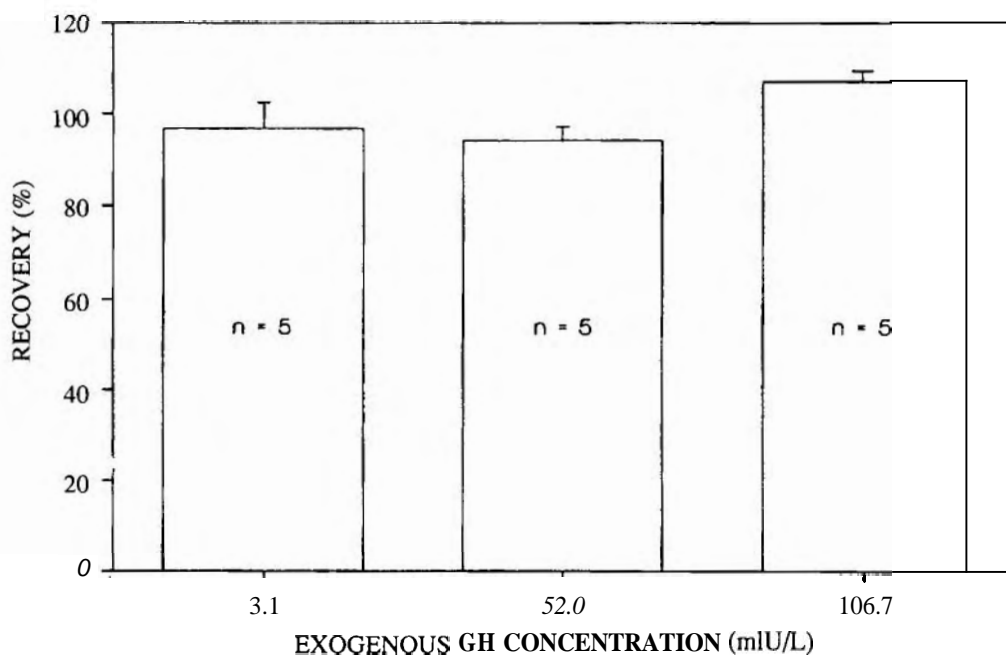


FIG. 5: Analytical recovery of in-house GH ELISA.

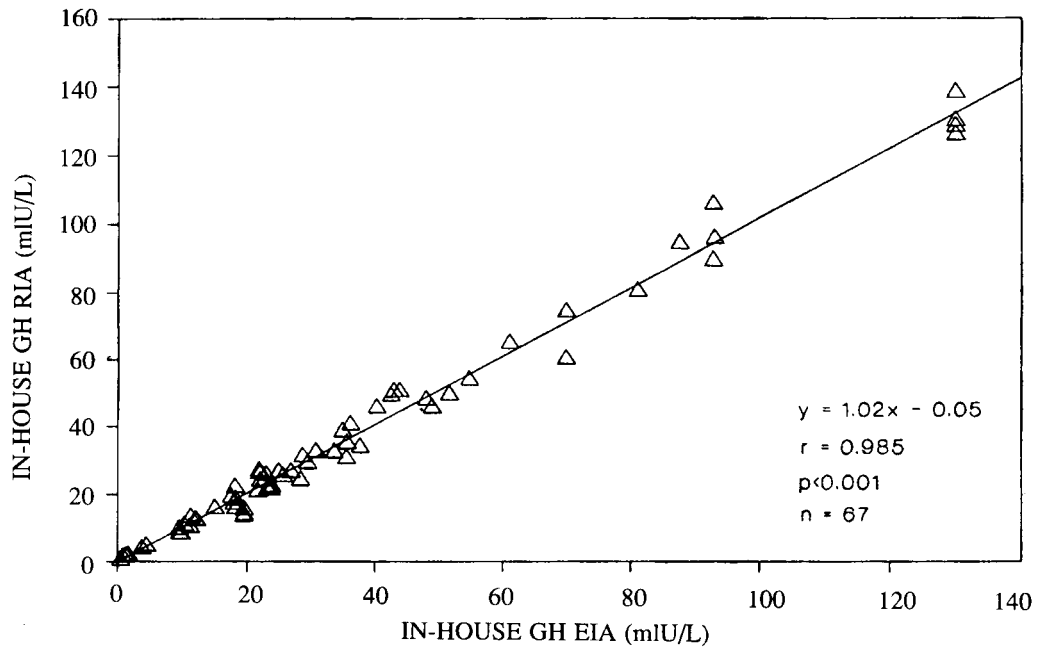


FIG. 6: Correlation of serum GH levels measured in in-house ELISA versus in-house RIA.

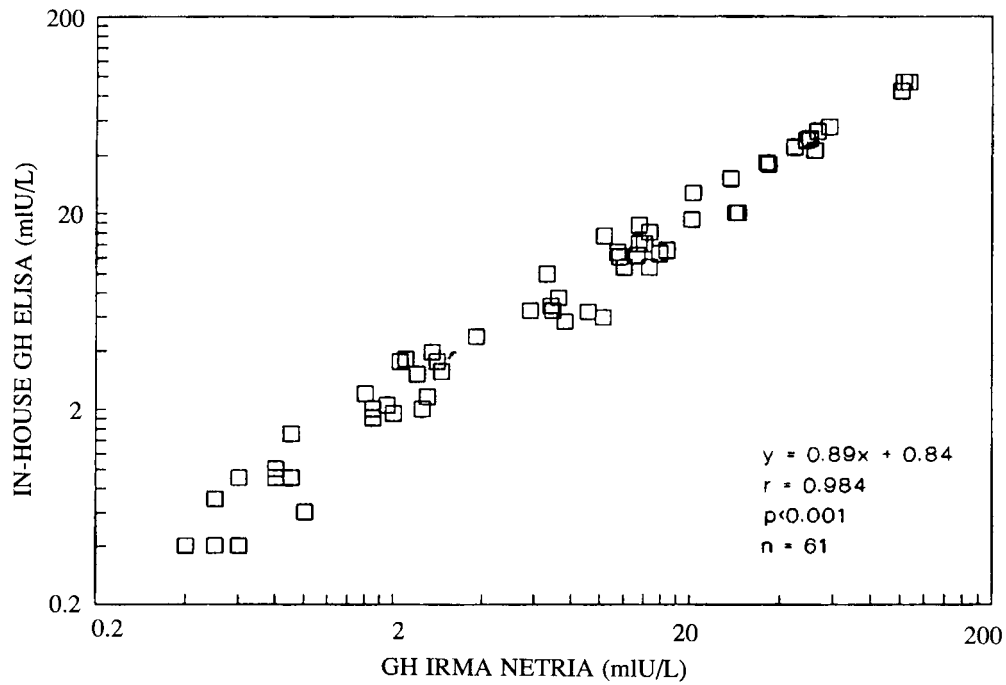


FIG. 7: Correlation of serum GH levels measured in in-house ELISA versus IRMA from NETRIA.

TABLE 1: Parallelism study of in-house GH ELISA.

Dilution	GH samples (mU/L)					% Mean recovery
	I	II	III	IV	V	
neat	25.9	46.8	49.9		53.5	68.5
1:2	12.3	24.1	24.4	24.5	37.0	96.5 ± 5.0
1:4	6.4	12.9	12.1	12.6	19.3	102.0 ± 8.6
1:8	3.3	6.6	6.4	6.4	10.0	105.6 ± 8.0

Serum samples were assayed neat, and after dilution with assay buffer at 1:2, 1:4 and 1:8

lipoproteins, pathogenic antigens and specific antibodies have been mainly by immunoassays. From the initial sensitive and specific isotopic methods of RIA and IRMA, there is now a widespread development of nonisotopic immunoassays using labeling substances such as enzymes, fluorescent or luminescent compounds.* These assays are widely available as commercial kits, although they can prove to be very expensive especially when used for routine diagnostic tests.

On the other hand, it is possible to develop a number of such immunoassay systems in-house, as have been shown in our previous¹² and current studies. Using our in-house assay, we were able to establish local reference values,¹⁴ enabling a more precise interpretation of results. With the use of single immunoassay method, it is also possible to standardise our results, thereby avoiding the problems faced when several immunoassay systems are used.^{9,10,15}

Our in-house ELISA was highly sensitive and specific, with very good recoveries of exogenous GH (95% - 106%) and parallelism of diluted samples (98 - 101%). It showed excellent reproducibility, with intra- and inter-assay CVs ranging from 4.8% - 8.7% at concentrations between 3.4 to 55.7 mIU/L. Good correlations between our established in-house RIA (r=0.985,

p<0.001) and the universally used IRMA from NETRIA (r = 0.984, p<0.001) further proved the validity of our in-house ELISA. In addition, with the wide working range of our ELISA standards (0.7 - 200 mIU/L), high GH levels such as from acromegalic patients, can now be quantitated directly without prior dilution. With the absence of matrix effect, we have also solved the problems of disparate results when standards are prepared in different diluent buffer.^{10,11} Compared to our previous reported in-house RIA,¹² which needed 100 ul of serum for analysis, the ELISA required only 20 ul serum, making it more suitable for use when blood sampling is a problem (such as in children). The small volume of standard/sample could be automatically dispensed with buffer, thereby, making our ELISA suitable for routine use in clinical laboratories.

Our assay procedure of coating the plate with PAB-GH (at 1:20,000) is simple and easy to perform when compared to the coupling of anti-GH to polystyrene beads in the EIA GH¹⁶ or the preparation of coupled polyclonal anti-GH-magnetic particles as in immunochemiluminometric assay.¹⁷ Although the fluorescence immunoassay reported by Kahan *et al.*¹⁸ was claimed to be highly sensitive (detection limit of ≤0.1 ug/L) with only 5 hours incubation, the method requires specialised and expensive equipment.

Our in-house ELISA is thus a good alternative immunoassay to RIA and IRMA. Although considerable time and effort were needed to develop and validate our in-house immunoassay, there are many advantages, such as low cost, and the avoidance of using radioactive materials.

REFERENCES

1. Gunn I. Growth hormone deficiency. *Ann Clin Biochem* 1987; 24: 429-34.

TABLE 2: Intra- and inter-assay imprecision of in-house GH

GH (mIU/L)	Intra-assay		Inter-assay	
	n	% CV	n	% CV
3.4	8	6.2	8	6.5
11.8	8	4.8	8	5.9
19.1	8	5.3	8	5.8
55.7	8	7.9	8	8.7

2. Linder B, Cassorla F. Short stature: etiology, diagnosis and treatment. *J Am Med Assoc* 1988; 260: 3171-5.
3. Thomer MO, Vance ML. Growth hormone. *J Clin Invest* 1988; 82: 745-7.
4. Nabarro PK. Acromegaly. *Clin Endocrinol (Oxf)* 1987; 26: 481-512.
5. Besser GM, Wass JAH. The pathophysiology of acromegaly. In: Molinatti GM and Martini L, eds. *Endocrinology '85*, Elsevier Science (Biomedical Division): 1989: 83-90.
6. Chard T. An introduction to radioimmunoassay and related techniques. Elsevier, Amsterdam, 1987.
7. Hunter WM, Bennie JG, Budd PS, *et al.* Immunoradiometric assays using monoclonal antibodies. In: Hunter WM and Come JET, eds. *Immunoassays for Clinical Chemistry*, Churchill Livingstone: 1983: 531-44.
8. Gosling JP. A decade of development in immunoassay methods. *Clin Chem* 1990; 36: 1408-27.
9. Reiter EO, Morris AH, MacGillivray MH, Weber D. Variable estimates of serum growth hormone concentrations by different radioassay systems. *J Clin Endocrinol Metab* 1988; 66: 68-71.
10. Celniker AC, Chen AB, Wert Jr RM, Sherman BM. Variability in the quantitation of circulating growth hormone using commercial immunoassays. *J Clin Endocrinol Metab* 1989; 68:469-76.
11. Felder RA, Holl RW, Martha Jr P, *et al.* Influence of matrix on concentrations of somatotropin measured in serum with commercial immunoradiometric assays. *Clin Chem* 1989; 35: 1423-6.
12. Wan Nazaimoon WM, Ng ML, Satgunasingam N, Khalid BAK. Development of an in-house radioimmunoassay for human growth hormone. *Malays J Pathol* 1990; 12(1): 13-20.
13. Vaitukaitis J, Robbins JB, Nieschlag, Ross GT. A method for producing specific antisera with small doses of immunogen. *J Clin Endocrinol* 1971; 33: 988-91.
14. Wan Nazaimoon WM, Ng ML, Satgunasingam N, Khalid BAK. Evaluation of suppression of growth hormone levels following a 75g oral glucose tolerance test. *Med J Malaysia* 1992; 47(2): 1-7.
15. Banfi G, Marinelli M, Casari E, Murone M, Bonini P. Isotopic and nonisotopic assays for measuring somatotropin compared: Re-evaluation of cutoff value in provocative tests. *Clin Chem* 1991; 37: 273-6.
16. Hashida S, Nakagawa K, Imagawa M, *et al.* Use of inorganic salts to minimize serum interference in a sandwich enzyme immunoassay for human growth hormone using Fab'-horseradish peroxidase conjugate. *Clin Chim Acta* 1983; 135: 263-73.
17. Weeks I, Woodhead JS. Measurement of human growth hormone (hGH) using a rapid immunochemiluminometric assay. *Clin Chim Acta* 1986; 159: 139-45.
18. Kahan I, Papanastasiou-Diamandi A, Ellis G, *et al.* Sensitive time-resolved fluorescence immunoassay of somatotropin in serum. *Clin Chem* 1990; 36: 503-8.