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ORIGINAL ARTICLES

A Supersensitive In-House Enzyme-linked Immunosorbent Assay (ELISA) for Measurement of Thyroid-Stimulating Hormone (TSH) and its Clinical Applications

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Summary

A supersensitive ELISA was developed for measurement of thyroid-stimulating hormone (TSH) concentrations in serum using in-house rabbit polyclonal antisera and a commercial monoclonal antibody. The assay was optimised and validated by recovery, linearity and cross-reactivity experiments and further compared to other available assays and EQAS samples. Good precision was obtained with a working assay range of 0.2 to 100 mIU/L with <10% coefficient of variation (CV) for both intra and interassay. The assay is highly sensitive and specific with a minimum detectable limit of 0.07 mIU/L and negligible cross-reactivities against LH, FSH, HCG and other pituitary peptides. Good correlations were obtained when compared to Abbott hTSH EIA ($r=0.993$; $p<0.001$; $n=85$) and NETRIA IRMA ($r+0.995$; $p<0.001$; $n=76$). The normal reference range established was 0.4 to 4.0 mIU/L ($n=76$). TSH levels in serum of thyrotoxic patients ($n=83$) were significantly lower (0.07 to 0.20 mIU/L, $p<0.0001$) and completely distinct from normal values thereby obviating the requirement of a TRH-stimulation test. Stability studies showed that coated wells can be stored at 4°C for at least 2 months. This highly sensitive in-house hTSH ELISA which is cheap, stable and readily available is useful for diagnosis and management of patients with various thyroid disorders.

Key words: ELISA, TSH, thyroid status

Introduction

The principal desirable characteristics of TSH assay that will distinguish normal subjects from those with hyperthyroidism include rapidity, convenience, high sensitivity and specificity. The use of various technical modifications of standard TSH radioimmunoassay (RIA) and immunoradiometric assay (IRMA) methods has improved sensitivity. Most of these methods require special and expensive instruments or involve laborious extraction techniques and radioactive labels[1,2]. The development of technology for immunoenzymometric assay[3,4] and the ability to produce specific monoclonal antibodies to TSH, have led to the generation of new assays with the potential to meet the above criteria. In this study

we developed a highly sensitive and specific ELISA for hTSH. The assay is easily performed and readily adaptable for use in any research or clinical diagnostic laboratory. We also report here the clinical applications of this assay.

Materials and Methods

Reagents

Commercial rabbit antibody to human TSH (Calbioche, Behring Diagnostics, La Jolla, CA 922037) and in-house rabbit antisera (Pcab 8901 and Pcab 8902) were diluted 2,000, 6,000 and 15,000-fold at working dilution with 15 mM Na_2CO_3; 35 mM $NaHCO_3$ buffer, pH 9.6. Mouse monoclonal antibody to human TSH (Serona Diagnostics, Norwell, A 02061) was diluted 21,000-fold from a concentration of 4.19 mg/ml to 0.2 µg/ml with a 1 g/L solution of bovine serum albumin (BSA, Sigma Chemical Company, Diesenhofe, FRG) in phosphate-buffered saline (PBS) pH 7.2, containing, per litre, 1.15 g of NaHPO, 0.2 g KHPO, 9 g NaCl and 0.01% (w/v) thimerosal as preservative. Affinity purified horseradish peroxidase labelled goat antiserum to mouse IgG (Bio-Rad Laboratories, CA) was diluted 3,000-fold with a 1 g/L solution of BSA in PBS buffer. The peroxidase substrate solution for horseradish peroxidase (HRP, EC11 11.1.7) was freshly prepared by dissolving 40 mg of o-phenylenediamine dihydrochloride (OPD, Sigma) in 100 ml of 50 mM Na_2HPO_4, 25 mM citric acid buffer, pH 5.0. Immediately prior to use, 60 µl of 30% H_2O_2 was added. Blocking solution was prepared by adding 10 g of BSA per litre of PBS buffer (1% (w/v) BSA). PBS containing 0.05% Tween 20 surfactant (v/v), pH 7.2 was used for washing the microtitre plates, PBS containing 0.1% BSA (w/v) was used as the assay buffer. TSH-free serum was prepared by passing pooled human serum through a column of charcoal-celite (Charcoal, 'NORIT OL' celite, 'Celite' 545, Hopkin and Williams, England) to remove TSH and other hormones. This charcoal-stripped human serum was used to dilute the TSH standard World Health Organization's 2nd International Reference Standard Preparation for hTSH (IRP 80/558) solution to concentration of 0.2 to 100 milliinternational units/L (mIU/L). Alternatively, sera from patients with overt hyperthyroidism were screened by replicate analysis in the in-house hTSH ELISA, and then pooled for use as 'TSH-free serum'.

Subjects

Basal TSH concentrations in sera of 76 normal adult healthy men and non-pregnant women were measured. Samples from patients were classified on the basis of clinical evaluation and serum levels of total thyroxine (T4) and triiodothyronine (T3) determined by radioimmunoassay (NETRIA RIA). Autoimmune thyroid diseases were excluded from the group of normal subjects by assaying for anti-thyroglobulin and anti-thyroid microsomal antibodies using our in-house thyroid autoantibodies ELISA immunodiagnostic test kits[5,6]. Samples from 97 euthyroid, 83 thyrotoxic and 95 hypothyroid patients from the Endocrine Clinic, Universiti Kebangsaan Malaysia were also assayed. All samples were stored at 20°C to –70°C prior to assay.

Production of rabbit antisera

Two rabbits were each injected intradermally (30-50 sites) with a total of 100 µg of purified TSH (code TSH M10) in complete Freund's adjuvant[7]. Following immunisation, the rabbits were bled from the marginal ear vein. Immunisation of rabbits with purified TSH yielded an antiserum with a titre of 1 in 725,000 (designated Pcab 8902) and 1 in 12,500 (designated Pcab 8901) when analysed in the Direct ELISA (titre definition: OD 492 nm+1.0 unit) and both giving a final working titre of 1 in 15,000 and 1 in 6,000 respectively.

Antibody specificity was determined by adding potentially cross-reacting glycoprotein hormones and other pituitary peptide hormones to serum-based zero calibrator and assayed. The percentage (%) cross-reactivity was calculated for the highest concentration of hormone tested. The equation used for calculating cross-reactivity was:

$$\frac{\text{measured TSH value (µg/L), apparent TSH response}}{\text{concentration of the cross-reacting analyte, µg/L}} \times 100\%$$

Cross-reactive hormones were assayed in duplicate of a series of serial dilution of each cross-reactant.

ELISA procedure

Rabbit antibody to human TSH (100 µl/well) was absorbed onto 96 well ELISA plates (Nunc-Immunoplate Maxisorp F96, A/S NUNC, Kamstrup, 4000 Roskilde, Denmark) for 4 hours (h) at room temperature (RT). Unbound sites in the wells were blocked with 300 µl of blocking solution per well for 2 h at RT. The plates were then flicked to semi-dryness and washed 3 times with 300 µl wash solution per well with NUNC-ImmunoWash (Nunc, Denmark). Standard or patient's serum (100 µl in duplicates) were then placed in each well and the plates were covered with plastic film and incubated overnight (16 to 18 h) at 4°C. After washing the plates, monoclonal antibody to TSH (100 µl per well) was then added to each well and allowed to react for 2 h at RT. After emptying and washing the plate, 100 µl goat anti-mouse IgG-horseradish peroxidase was added. The plate was incubated at RT for 1 h, then emptied, washed 5 times and 100 µl of the substrate for horseradish peroxidase added to each well. After 20 min incubation at RT, the enzymatic reaction was terminated by adding 50 µl of 1.25 M sulphuric acid. The absorbance was read immediately with an automatic AT 400 SLT Easy Reader (SLT-Labinstruments, Groedig/Salzburg, Austria) using the dual wavelength mode, 492 nm minus reference 620 nm. The absorbances were either manually or automatically transferred to a LKB-Wallac RIA-Calc program (Wallac Oy, Turku, Finland) for curve fitting and data analysis.

Recovery

Recovery was carried out using either normal, euthyroid and thyrotoxic serum samples spiked with 0.2, 2.0, 12.5, 50 and 100 mIU/L of TSH. Recovery was calculated by expressing the net TSH (measured–initial TSH concentration) determined by ELISA as a percentage of added TSH.

Table I
Cross-reactivity of glycoprotein and other pituitary peptide hormones in hTSH ELISA in-house assay

Crossreactivity Hormones	Pcab 8901		Pcab 8902	
	%	Highest concentration tested (µg/L)	%	Highest concentration tested (µg/L)
hTSH	100		100	
Beta-hTSH	38.8	22	40.2	30
Alpha-hTSH	2.1	320	2.7	400
h-FSH	0.9	2,000	0.7	2,000
Beta-hFSH	<0.10	2,800	<0.10	2,500
hLH	<0.10	7,000	<0.10	6,500
Beta-hLH	<0.10	5,000	<0.10	4,600
hCG	<0.10	12,000	<0.10	10,000
Beta-hCG	<0.01	20,000	<0.01	17,000
hGH	<0.10	8,000	<0.10	6,000
hPL	<0.01	200,000	<0.01	200,000
hPRL	<0.10	1,800	<0.10	2,000

Pcab 8901 and Pcab 8902 are in-house rabbit polyclonal antisera.

Parallelism

Dilution studies were carried out using 3 hypothyroid specimens serially diluted with the serum-based zero calibrator. TSH levels were assayed using ELISA and calculated as percentage (%) recovery.

Precision

Intra-assay variation was calculated from the differences between 6 sets of duplicates using human serum samples. The inter-assay variability was determined by performing duplicate measurements in consecutive assays on 6 different days.

Immunoradiometric assay (IRMA) and enzyme immunoassay (EIA)

For comparison purposes, TSH was measured by the IRMA method routinely used in our laboratory, which employs sheep anti-TSH antiserum and a mouse monoclonal anti-TSH 125 I-labelled antibody (North-east Thames Region Immunoassay Unit, NETRIA). Commercial enzyme immunoassay Abbott hTSH EIA diagnostic test kits using monoclonal antibodies directed against different antigenic sites of hTSH were purchased from Abbott Laboratories, North Chicago, IL.

Statistical analysis

The Chi squared goodness-of-fit test was used to assess the normality of distribution of log transformed TSH values obtained for normal subjects[8]. Pearson's correlation coefficient (r) was used to show the degree of linear association among the different variables and Student's t-statistics for comparison of means. A p value of <0.05 was considered significant.

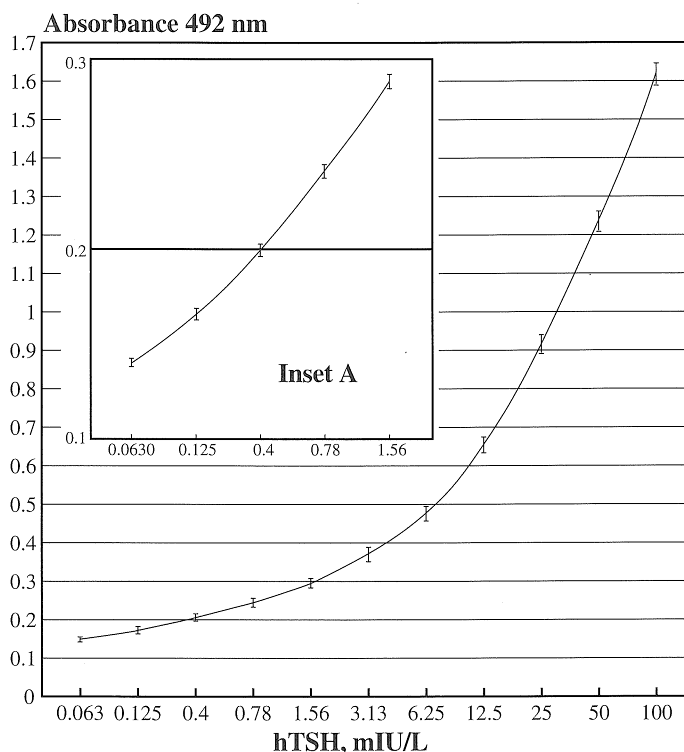


Fig 1: Typical standard curve for in-house hTSH ELISA assay. Points (and bars) represent means (±SEM) for triplicate determinations. Inset A shows a plot of the 5 lowest standards to aid in interpolation of low TSH concentration

Results

Polyclonal antibody specificity

To evaluate cross-reactivities towards hCG, LH, FSH and other pituitary peptide hormones in the in-house ELISA assay system, serial dilutions of the appropriate hormones were added to TSH-free serum and assayed. Rabbit anti-hTSH polyclonal antibodies had 40% cross-reactivity to beta-subunit TSH, 2% alpha-subunit TSH, (1% FSH and 0.1% LH/HCG/HGP/ZPRL (Table I).

Assay performance

Fig 1 shows the standard curve for sensitive ELISA for human TSH. The lowest 5 standards were replotted on a larger scale for easier interpolation of low TSH concentrations (see inset A). The minimal detectable limit, calculated as 3 standard deviations (SD) from zero standard was 0.07 mIU/L. The 'hook' effect, due to excess antigen[9] and characteristic of sandwich-type assays[10], occurred at about 250 mIU/L for commercial polyclonal antibody (Cpcab), and about 500 mIU/L for both in-house polyclonal antibody, Pcab 8901 and Pcab 8902 (Fig 2).

Analytical recovery and linearity

For the hTSH ELISA in-house assay using commercial antibody (Cpcab), recoveries of added TSH ranged from 93% to 109%, mean of 101.3%. The corresponding recoveries using Pcab 8901 ranged from 91% to 109% with mean of 98.4% and recovery ranged from 93% to 108%, mean of 100.3% for Pcab 8902.

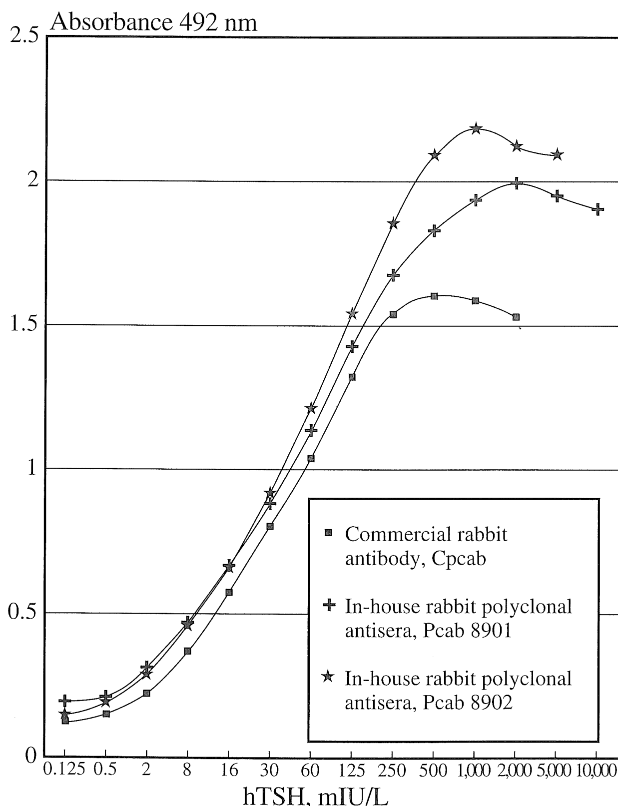


Fig 2: The dose-response curve for TSH ELISA prone to high dose 'hook' effects at some point. The shape of the standard curves begin to decline with increasing TSH concentration after reaching a maximum. Each point represents the average value of duplicate assays

Table II
Precision of hTSH ELISA

TSH (mIU/L)	Intra-assay		Inter-assay	
	n	*CV(%)*	*n*	*CV(%)*
Cpcab				
0.5	6	8.8	6	9.4
2.0	6	5.9	6	7.2
45.0	6	3.6	6	5.3
Pcab8901	<0.10	2,800	<0.10	2,500
0.5	6	9.2	6	9.8
2.0	6	7.0	6	7.4
45.0	6	4.1	6	5.6
Pcab8902	<0.01	20,000	<0.01	17,000
0.5	6	8.4	6	8.8
2.0	6	6.3	6	6.9
45.0	6	3.8	6	5.1

Cpcab : Commercial rabbit antibody. Pcab 8901/8902 : in-house rabbit antisera.

For assessment of dilution linearity, the concentrations of hTSH in the undiluted samples were used to calculate the expected values of the diluted samples. Excellent agreement between results for diluted samples versus expected values were obtained which ranged from 92.3% to 109.0% for commercial polyclonal antibody (Cpcab); 90.0% to 107.9% for Pcab 8901 and 92.6% to 108.1% for Pcab 8902.

Precisions

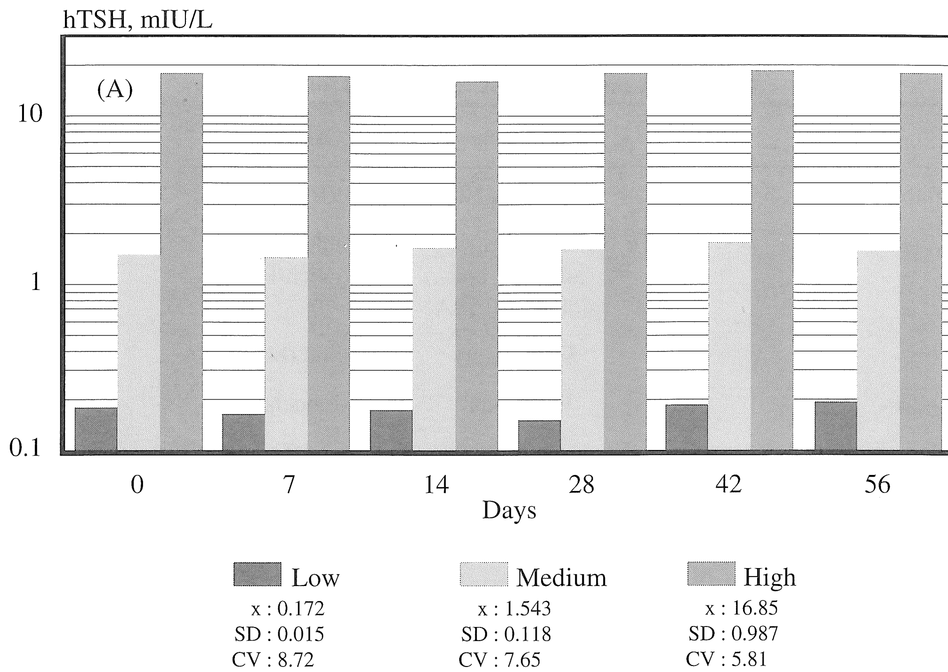
Intra and inter-assay coefficients of variation (CVS) ranged from 3.6% to 8.8%, 4.1% to 9.2% and 3.8% to 8.4% respectively for Cpcab, Pcab 8901 and Pcab 8902 for TSH values of 0.5 to 45.0 mIU/L (Table II). The corresponding inter-assay CVs were all <10% (Table II).

Stability of coated microtitre plates

The criterion used for coated microtitre plates stability was the run-to-run precision of the assay. If results obtained for a 2 month period remained within the run-to-run precision (a CV of <10%), they were considered to be the same as initial assay values. Coated microtitre plates (using polyclonal antibody Pcab 8901 and Pcab 8902) stored dry at 4°C after being blocked with 1% BSA/PBS, were stable for a minimum of 2 months as shown in Fig 3.

Comparison with Abbott hTSH EIA and Netria IRMA

Table III lists the characteristics of the in-house ELISA, Abbott EIA (non-isotopic method) and NETRIA IRMA (isotopic method) assays, including the calibration materials (International Reference Preparations). Results of the in-house ELISA assay were compared with those by the Abbott hTSH EIA commercial kit and NETRIA IRMA. The correlations between results of the in-house ELISA assay (using in-house rabbit polyclonal antisera or commercial rabbit antibody) versus those by Abbott hTSH EIA (Figures 4A and B) and NETRIA IRMA were all excellent ($r>0.90$, $p<0.001$) as summarised in Table IV. The Altman-Bland plot of mean difference against mean of the 2 methods allows one to investigate any



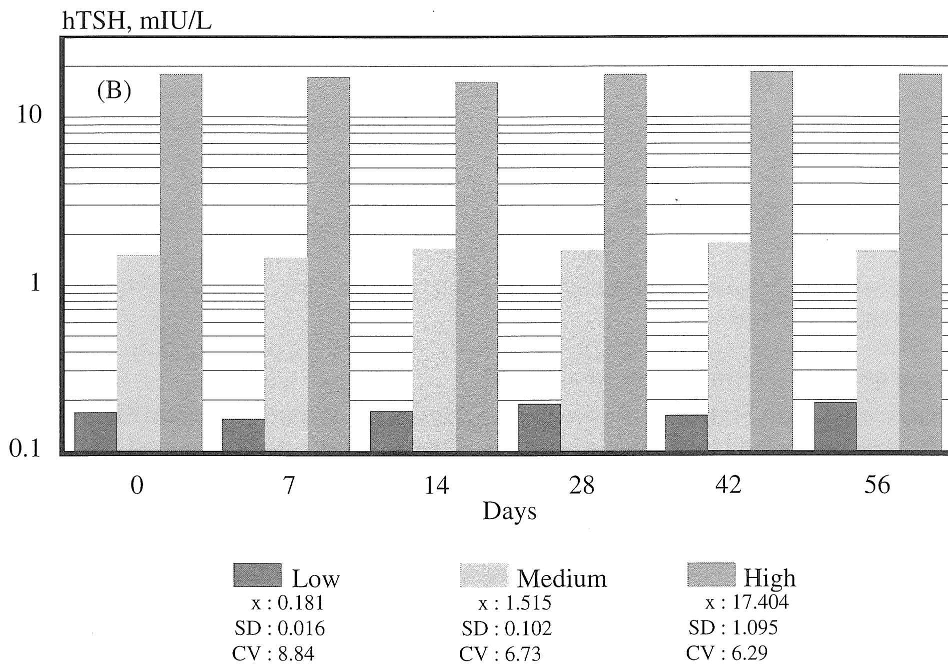


Fig 3: Effects of storage temperature and duration on coated microtitre plates using in-house rabbit polyclonal antisera, Pcab 8901 (A) and Pcab 8902 (B). Each concentration (low, medium and high) is the average of 3 individual samples, assayed in duplicates

Table III
Main characteristics of the hTSH ELISA in-house assay, Abbot EIA and NETRIA IRMA used for TSH assay

	In-house assay	Abbott	NETRIA
Calibrator	IRP 80/558	IRP 80/558	IRP 80/558
Assay method	Double antibody sandwich-anti-globulin ELISA	Double antibody sandwich-ELISA	2-site IRMA
Phase	Solid (well)	Solid (bead)	Solid (tube)
Antibodies	Polyclonal	Monoclonal	Polyclonal
Sample volume, µl	100	100	100
Wash steps	6	2	2
Sample incubation time, h	O/N[d]	1	O/N
Incubation temperature	RT	37°; RT (substrate solution)	RT
Standards Range, mIU/L	0.07-100[a]	0.05-60	0.15-60
Matrix (serum)	Human	Human	Human
Minimum detectable limit (MDL), mIU/L	0.07[c]	0.05[b]	0.15[c]

a : working range of 0.2 to 100 mIU/L with 10% CVs for both intra-assay and inter-assay
b : from manufacturer's insert (the 0 mIU/L Standard was assayed in replicates of 10 in 5 separate assays; 95% confidence limit)
c : minimum concentration that can be distinguished from zero at 99.73% confidence level (3SD), and
d : the overnight incubation step, while not mandatory, allows assay work-load to be spread evenly

possible relationship between the measurement error and the true value. The mean of the 2 measurements is the best estimate of true value[11].

External quality assessment scheme (EQAS)

Correlation between hTSH ELISA in-house assay versus ALL Laboratory Trimmed Mean (AALTM) of EQAS samples, organised by International Atomic Energy Agency (IAEA) showed highly-significant correlation ($r=0.989$, $p<0.001$, $n=30$).

Clinical performance of the hTSH ELISA in-house assay

Serum was obtained from blood samples of 76 normal volunteers, 97 euthyroid, 83 hyperthyroid and 95 hypothyroid patients. In normal subjects, TSH concentrations showed a on-Gausian distribution (Fig 5A). The normal reference interval was derived from the mean+2SD (95% confidence limits) of logarithmically transformed values which ranged from 0.4 to 4.0 mIU/L (antilog mean, 1.7 mIU/L). It has been shown previously that sex-related differences in basal TSH concentrations are not statistically significant[12,13]. The TSH concentrations in all thyrotoxic patients (<0.07 to 0.20 mIU/L) (Fig 5B) were significantly lower ($p<0.001$) than normal (Table V). These findings indicate that all of thyrotoxic patients

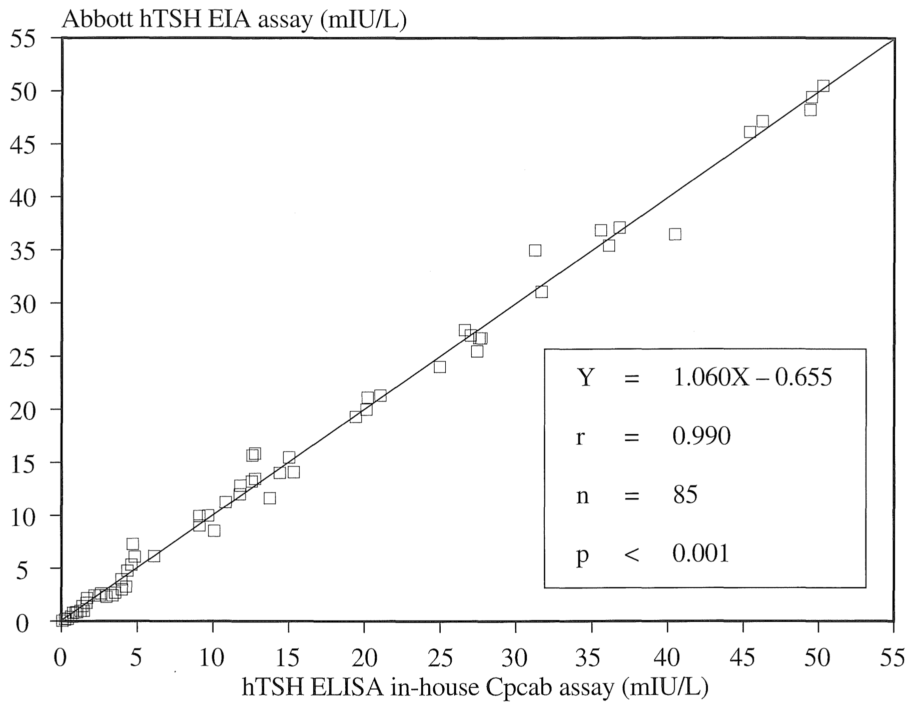


Fig 4A: Correlation of hTSH ELISA in-house using commercial rabbit antibody (Cpcab) versus Abbott EIA

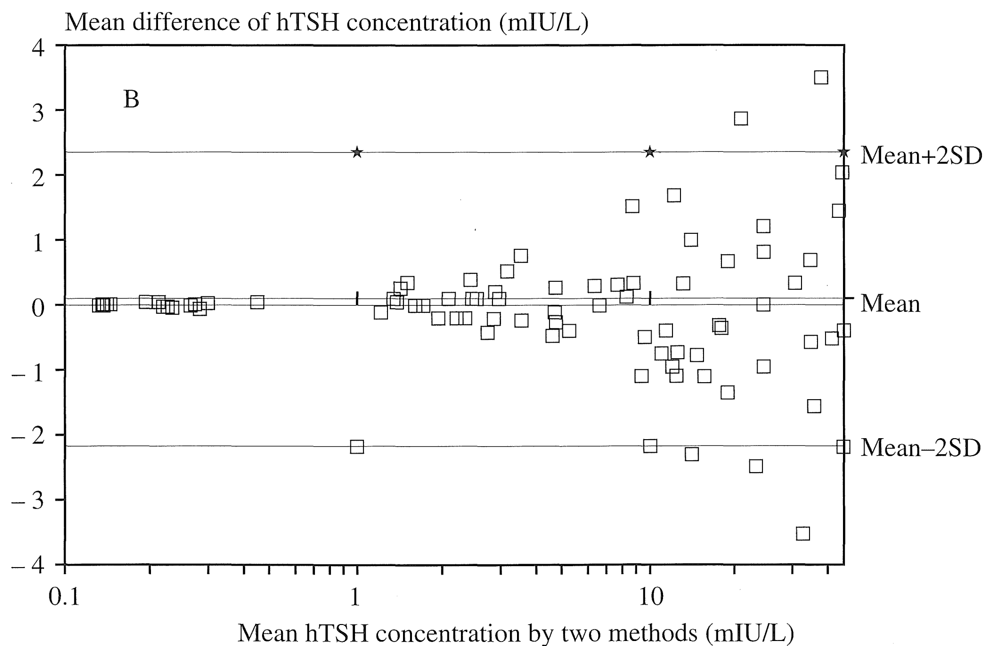


Fig 4B: Altman-Bland plot of mean difference hTSH by hTSH ELISA in-house Cpcab and Abbott EIA versus mean of the 2 methods

Table IV
Method comparison studies with clinical samples

Comparison	n	Intercept, mIU/L	Slope	r
Cpcab vs Abbott EIA	85	–0.655	1.060	0.990
Cpcab vs NETRIA IRMA	76	0.319	1.010	0.993
Cpcab vs Pcab 8901	46	–0.099	1.021	0.966
Cpcab vs Pcab 8902	46	–0.338	1.009	0.966
Pcab 8901/02 vs Abbott EIA	46	0.317	0.947	0.925
Pcab 8901/02 vs NETRIA IRMA	46	0.655	0.962	0.938

Cpcab: commercial rabbit antibody. Pcab 8901 and Pcab 8902: in-house rabbit polyclonal antisera, r: correlation of coefficient. Samples were assayed in duplicate. The samples used in the linear regression analysis were obtained from normal, euthyroid, hypothyroid, and hyperthyroid individuals.

Table V
Mean and range (+2SD) of TSH, T_3 and T_4 concentration in sera from normal, hypothyroid, hyperthyroid and euthyroid persons.

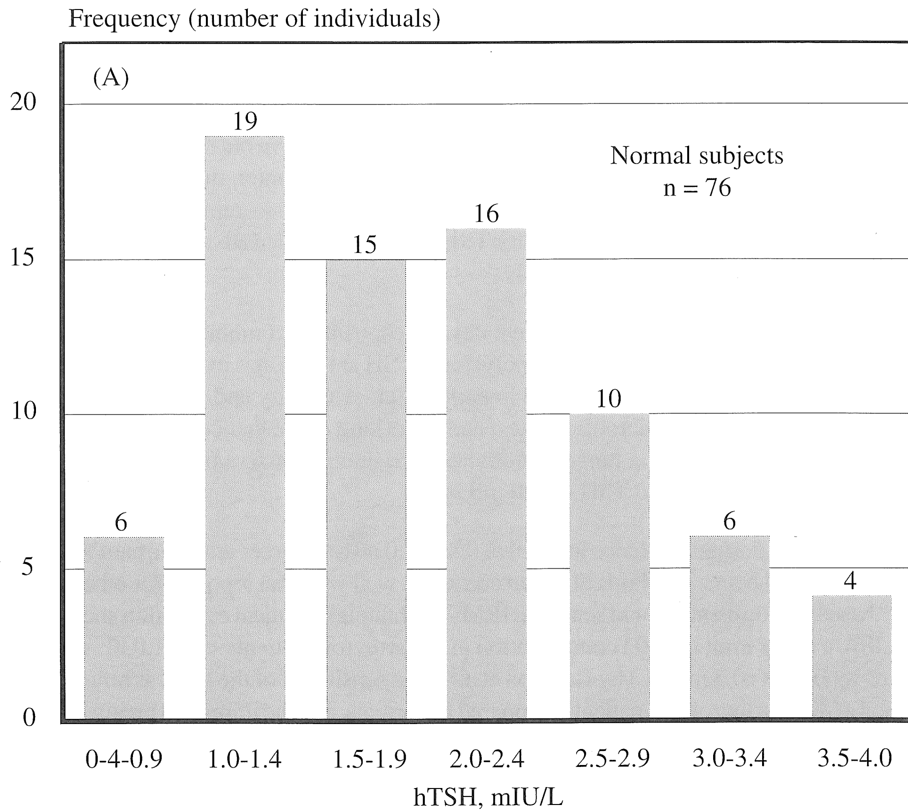
Group	hTSh, mIU/L Mean	hTSh, mIU/L Range	T_3, nmol/L Mean	T_3, nmol/L Range	T_4, nmol/L Mean	T_4, nmol/L Range
Normal (n=76)	1.9	0.4-4.0	2.00	1.05-2.95	102	50-154
Euthyroid (n=97)	2.3[a]	0.4-4.5	2.15	1.15-3.15	112	62-162
Hyperthyroid (n=83)	0.09[b]	<0.09-0.20	4.85	3.20->10	204	165->250
Hypothyroid (n=95)	28.6[b]	10-100	1.05	0.60-1.80	51	30-68

a: no statistical difference from normals, and b: $p<0.001$ compared to normals

can be distinguished from normal on the basis of a single measurement of the TSH concentration. Of the 95 hypothyroid patients, all had above-normal values for TSH ($p<0.001$), in the range of 8 to 100 mIU/L (Table V).

Discussion

Conventional RIAs for human TSH will reliably discriminate between the low values seen in normal subjects and the above normal ones characteristic of primary hypothyroidism. However, concentrations of TSH in serum from patients with either pituitary hypofunction or hyperthyroidism of most aetiologies are undetectable in such assays, and thus not clearly distinguishable from normal. Problems inherent in RIA methodology include the immunological and radiochemical instability of human TSH after ^{125}I labelling by the Chloromine T technique with consequent increasing 'damage' that further reduces sensitivity and augments interassay variation. Moreover, effects of serum on precipitability of bound labelled antigen by second antibody have been described[14].



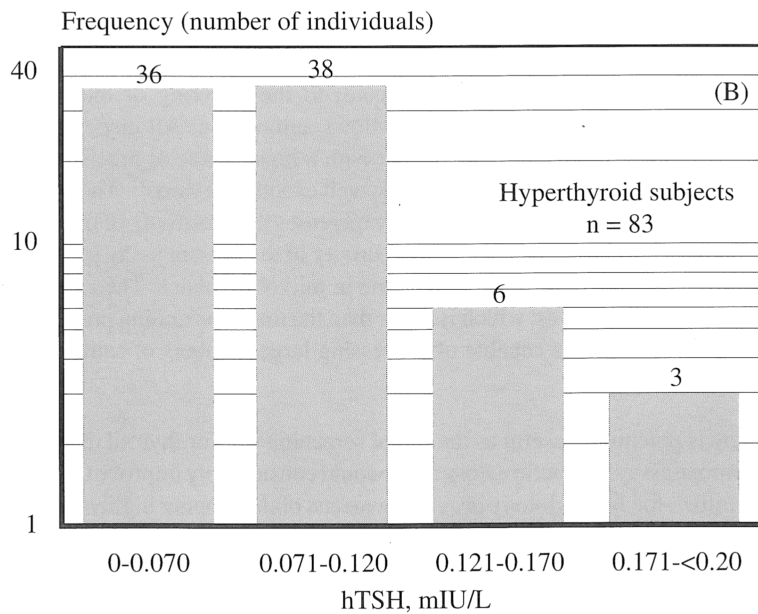


Fig 5: Distribution of TSH concentrations among normal subjects (A) and thyrotoxic patients (B)

Immunochemiluminometric assay (ICMA) involves reactions of serum samples with monoclonal antibodies to TSH that have been labelled with a chemiluminescent ester. Subsequent reaction with a solid-phase polyclonal antibody to TSH is followed by measurement of chemiluminescence in a luminometer. ICMA required luminometric measurement of photon emission, which is tedious and time dependent. Immunoradiometric assays using labelled antibodies are more sensitive but the need to use radioisotopes with their limited shelf-life, health biohazard, expensive gamma-counter, time-consuming for counting of tubes and attendant problem of disposal of ^{125}I-labelled materials — remain a significant drawback. Compared with other sensitive RIAs/IRMAs for TSH, ELISA has added advantages with regard to safety and the reagent stability of non-isotopic labels.

The hTSH ELISA in-house assay that we have developed, using a combination of mouse monoclonal antibody to beta-subunit of human TSH and polyclonal TSH antisera, has excellent inter and intra-assay precision; very good analytical recovery and linearity; high-sensitivity and specificity; storage stability of polyclonal antibody-coated microtitre plates; and a working range that covers the entire pathophysiological range for TSH, a working range that obviates pre-dilution of sera from hypothyroid patients in all but extreme cases of increased TSH (>100 mIU/L).

The in-house ELISA normal reference interval of 0.4 to 4.0 mIU/L agrees with Durham's proposal for an upper limit of normal being 3 or 4 mIU/L[15] and compares well with that reported for other sensitive TSH assays[16-19] based on non-isotopic and sensitive IRMA techniques. Clinical evaluation showed that the in-house ELISA's upper limit of TSH concentration in hyperthyroid patients <0.2 mIU/L was comparable to the value reported (<0.3 mIU/L) by Carayon et al[20]. The sensitivity of the assay is reflected in the clear suppression of TSH in thyrotoxic patients compared to normals, and the improved precision profile at low TSH concentrations as compared with published findings for some of the newer TSH assays[21]. Results of TSH assayed by the in-house ELISA were highly-correlated with the results for Abbott hTSH EIA commercial kit (r=0.925) and NETRIA IRMA (r=0.938). Analysis of external quality assessment scheme samples also showed an excellent correlation (r=0.989) with the All Laboratory Trimmed Means.

Assays using microtitre plates require careful handling of the plates in the difference analytical steps. In our experience, the washing procedure is especially important (e.g. adequate washing and aspiration to remove unbound antigen/antibody/enzyme conjugate) to the lowering of the non-specific binding background. The use of the automatic processor allows automation. All dispensing steps except the addition of standards or samples can be carried out with a high degree of precision (CV<1%) and the comprehensive wash and aspirate system treat every well exactly the same[22]. The linked enzyme serves to amplify the substrate colour change, which greatly increases the sensitivity of the assay such that a low range of TSH value can be measured. The high-sensitivity of the present assay permitted the use of 100 µl as the sample volume and for the assay to be done in microtitre plates. The ELISA reader is able to measure <1 min per plate of 96 wells, which is faster than the time-consuming procedures for radiostope quantification. This ELISA is thus capable of processing large numbers of samples used in screening programmes.

The in-house assay is potentially useful as the initial screening test for thyroid disease. This assay with other selected hormone assays as confirmatory tests, would considerably improve the efficiency of thyroid function testing further, the methodology provides a means of developing highly sensitive, non-isotopic test procedures for a wide range of glycoprotein and other pituitary peptide hormones. The assay is easy to set up: requires only simple readily available laboratory equipment, and reagent costs are low. The present ELISA has additional advantages in that radioactive materials are not used and that reagents have long shelf-lives under appropriate storage conditions. Since multiple samples can be analysed simultaneously, this technique has outstanding potential for routine diagnostic evaluation of patients with suspected thyroid dysfunction.

Acknowledgements

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