CLIN. CHEM. 32/3, 511–514 (1986)

New Immunoenzymatic Assay for Human Thyrotropin Compared with Two Radioimmunoassays

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We have evaluated a new immunoenzymatic assay for human thyrotropin involving three monoclonal antibodies (Abbott HTSH EIA) and compared the results with those of two conventional nonequilibrium double-antibody radioimmunoassay (RIA) methods: Clinical Assays' RIA and a research RIA (J Clin Endocrinol Metab 1975;41:676). Mean values for thyrotropin in 100 euthyroid serum samples were similar in the Abbott and Clinical Assays methods, but both sets were significantly higher than those by the research RIA. By all methods, values for hypothyroid patients were clearly higher than values for euthyroid subjects. Results for hyperthyroid and euthyroid subjects were resolved slightly better with the research RIA than with the Abbott kit. The new Abbott assay was far more sensitive than either the Clinical Assays RIA or our research RIA. The correlation of results of the Abbott assay with those of the Clinical Assays and the research RIA exceeded 90% for samples from hypothyroid patients. The Abbott assay replaces radioisotope counting with spectrophotometric detection.

Additional Keyphrases: *"kit" methods · intermethod comparison · thyroid status · monoclonal antibodies*

The development of highly sensitive ^{125}I-labeled assays for thyrotropin (TSH) over the past 10 years has promoted major advances in our understanding of the regulation of thyrotropin secretion in health and disease (*1*). Nevertheless, because of the accumulating burden of regulations concerning the acquisition, storage, distribution, handling and disposal of ^{125}I-labeled materials, the need for alternative methods for assaying TSH has become compelling. Ideally, these new methods will not sacrifice the best characteristics of the current state-of-the-art assays: high sensitivity, excellent reproducibility, short incubation times, and storage stability of tracer for at least two months.

We report here the results of our comparison of a new, state-of-the-art, nonisotopic method for measuring human TSH with high sensitivity (Abbott immunoenzymatic assay); a representative commercial (Clinical Assays) kit assay for TSH based on the standard nonequilibrium, double-antibody radioimmunoassay technique; and a similar research radioimmunoassay optimized for sensitivity rather than for minimum incubation time.

Materials and Methods

Abbott HTSH EIA. The thyrotropin (thyroid-stimulating hormone, TSH) diagnostic test kit (Abbott HTSH EIA) was provided by Abbott Laboratories, North Chicago, IL. This solid-phase immunoenzymatic method is based on the "sandwich" principle and involves three monoclonal antibodies directed against three different antigenic sites on the human TSH molecule. Two TSH-specific antibodies (one specific to the beta-subunit and one to intact TSH) are immobilized on a polystyrene bead. The alpha subunit-specific antibody, which is conjugated with horseradish peroxidase (EC 1.11.1.7), is supplied by the manufacturer in 0.1 mol/L Tris buffer, pH 7.5, with protein stabilizers and thimerosal (100 mg/L) as preservative. The complete composition of the substrate solution was not revealed by the manufacturer. The enzyme substrate solution, containing hydrogen peroxide and *o*-phenylenediamine (OPD), is added to the washed beads. In the presence of TSH, a yellow-orange color develops that is directly proportional to the quantity of TSH in the sample.

The assay procedure is as follows. Add 100 μL of standard, control, or specimen to each well of the narrow-well reaction tray supplied with each kit. Add one antibody-coated bead to each well, apply the cover seal, and incubate in a 37 °C water bath for 1 h. Remove and discard the cover seal, then wash the beads three times in distilled water with "Pentawash" (a bead-washing device supplied by the manufacturer). Add 200 μL of the TSH antibody–horseradish peroxidase conjugate to the beads, apply a new cover seal, and incubate 1 h at 37 °C as before. Remove and discard the cover seal. Wash the beads three times with Pentawash, then transfer the beads to 12 × 75 mm plastic test tubes. Pipet 300 μL of OPD substrate solution into each tube and into two empty tubes used for substrate blanks, and incubate at room temperature (15 to 30 °C) for 30 min. Pipet 1.0 mL of 1 mol/L sulfuric acid into each tube to stop the reaction, and vortex-mix. Measure the absorbance at 492 nm. Calculate the results by graphical or least-squares interpolation from a linear plot of A_{492} vs TSH concentration for the assay calibrators. [Abbott also has available a computerized spectrophotometer ("Quantum" analyzer) that provides complete data analysis for each sample inserted.]

Clinical Assays hTSH radioimmunoassay. The GAMMA-DAB® human serum [^{125}I]hTSH radioimmunoassay kit (Clinical Assays, Cambridge, MA) is a liquid-phase nonequilibrium radioimmunoassay in which a rabbit polyclonal antibody to human TSH is used. The assay procedure is as follows. Add 200 μL of calibrator, control, or unknown in duplicate to 12 × 75 mm test tubes. Add 100 μL of TSH antibody, then shake the samples for 15 s. Incubate for 18–24 h at room temperature, add 100 μL of ^{125}I-labeled TSH, and shake again for 15 s. Incubate for 3 h at 37 °C, then add 1 mL of goat antirabbit antiserum in dilute polyethylene glycol and incubate for at least 5 min in a 37 °C water bath. After centrifuging the samples (1000 × g, 15 min), decant the supernates and count the radioactivity of the precipitates for 1 min. Plot the ratio of the counts at finite concentration of TSH calibrator to the counts bound with the zero concentration of TSH calibrator vs the logarithm of the TSH concentration. Calculate unknown concentrations by graphical interpolation, or use a conventional radioim-

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Received September 9, 1985; accepted December 2, 1985.

munoassay analysis program to estimate the concentration of TSH in serum samples.

In-house "research" radioimmunoassay. For comparison, we correlated the Abbott kit results and those of the Clinical Assay kit with those obtained with a previously described (2) highly sensitive, nonequilibrium, double-antibody radioimmunoassay (VA TSH) as recently modified: we now incubate overnight at 22 °C 100 μL of calibrators and serum samples with 100 μL of rabbit antiserum to TSH (100 000-fold working dilution) in a total incubation volume of 0.4 mL. We then add 100 μL of ^{125}I-labeled TSH (6000 counts/min), incubate for 5 h at 22 °C, then add 100 μL of goat antiserum to rabbit gamma-globulin, and incubate overnight at 4 °C. After centrifuging (1000 × *g*, 30 min), we aspirate the supernatant fluid and determine the radioactivity in the pellet without further processing.

Results

The Abbott TSH values for a group of 100 euthyroid subjects did not differ from corresponding results obtained with the Clinical Assays kit (Table 1). However, the results from both of these assays were significantly higher than those obtained in the VA TSH assay ($p < .001$, two-tailed paired *t*-test). The Abbott data for 40 hypothyroid patients were significantly higher than the corresponding results for these 40 subjects measured with the Clinical Assays kit ($p < .001$) and in the VA TSH assay ($p < .05$, two-tailed paired *t*-test).

Assays of 40 sera from hyperthyroid patients (all with undetectable TSH in the VA TSH assay, <0.3 milli-int. unit/L) by the Abbott kit, showed that TSH was undetectable in 29 (<0.05 milli-int. unit/L) and was 0.4 (SD 0.1) milli-int. unit/L for the other 11 samples. The minimum detectable dose was calculated from the average of 2 SD at zero dose of TSH (0.00148 A_{492}) divided by the average slope of the standard curve at zero dose (0.031 A_{492}/milli-int. unit of TSH per liter). TSH was undetectable (<1.0 milli-int. unit/L) in 21 of the sera from 40 hyperthyroid patients when assayed with the Clinical Assays kit; the remaining 19 samples had 1.3 (SD 0.3) milli-int. unit/L. If we used the VA TSH assay minimum detectable dose of <0.3 milli-int. unit/L for both the VA and the Abbott assays, then two of the 100 euthyroid TSH results were undetectable by the VA TSH assay and three were undetectable by the Abbott kit.

Figure 1 depicts representative standard curves for the Abbott, Clinical Assays, and VA methods for measuring human TSH.

We determined within- and between-assay CVs for all three assays (Table 2). Hormone cross reactivities in the various methods are given in Table 3.

Regression analysis for TSH concentrations as measured with the Abbott (y) and Clinical Assays (x) kits gave $y = 1.029x + 0.018$ ($r = 0.71$, n = 94) for euthyroid results obtained in five consecutive Clinical Assay analyses and six consecutive Abbott kit assays. Regression of the Abbott kit (y) data vs the VA TSH (x') results for 94 euthyroid subjects gave $y = 0.903x' + 0.331$, $r = 0.68$. The corresponding regression analysis for results from 40 moderately to severely hypothyroid subjects is shown in Figure 2.

Table 1. Concentration of Thyrotropin in Serum as Measured by the Three Methods

	Mean (and SD) TSH concn, milli-int. units/L		
n	Abbott	Clinical Assays	Vet. Admin.
Euthyroid			
100	2.8 (2.0)	2.7 (1.4)	2.2 (1.5)
Hypothyroid			
40	94 (82)	71 (73)	81 (63)
Hyperthyroid			
40	ND in 29[a]	ND in 21[b]	ND in 40

[a] ND, not detectable (<.05 milli-int. unit/L). Remaining 11 values: 0.4 (SD 0.1) milli int. unit/L.
[b] ND (<1.0 milli-int. unit/L). Remaining 19 values: 1.3 (SD .3) milli-int. unit/L.

Discussion

The Abbott immunoenzymatic assay for human TSH incorporates several recent advances in TSH assay methodology. The use of monoclonal antibodies ensures a large supply of TSH-specific immunoglobulin of constant epitope specificity (3). The coupling of the TSH beta-subunit-specific immunoglobulins to the polystyrene bead permits quantitative removal, after the first incubation (4), of hormones structurally related to TSH, as well as other serum proteins which are responsible for the inhibitory "matrix" or "serum" effect on tracer binding (2). The horseradish peroxidase–alpha-subunit-specific immunoglobulin conjugate, added in the second incubation, binds to the intact, bead-immobilized TSH molecules (1). The remarkable improvement in assay minimum detectable dose, 0.05 milli-int. unit/L, as com-

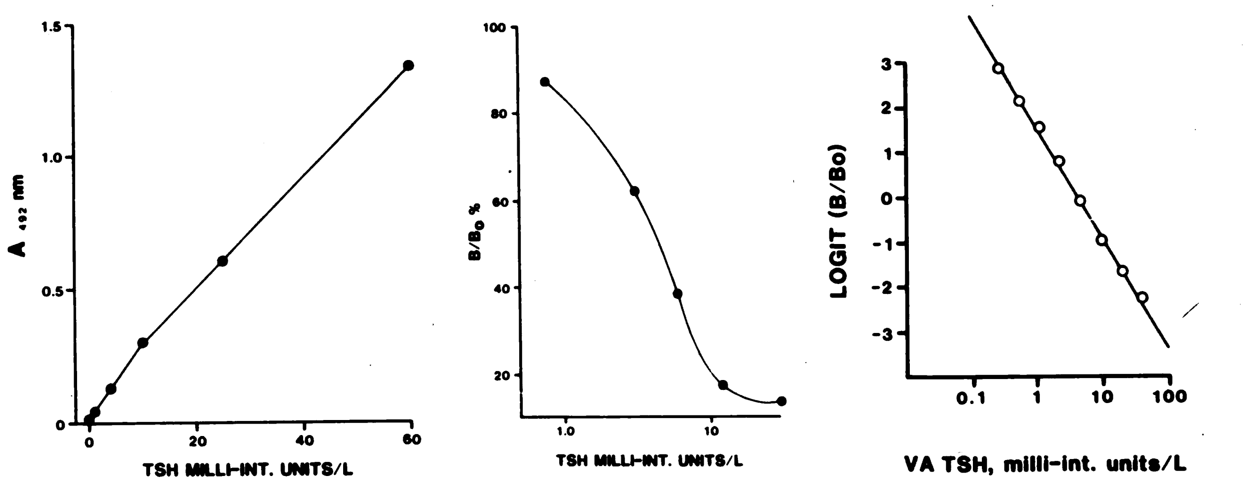

Fig. 1. Representative standard curves for (*left*) the Abbott TSH standard curve, (*center*) Clinical Assays TSH RIA: [B_0/T = 100% × counts bound at zero dose − nonspecific counts)/(total counts − nonspecific counts) = 30.2%], and (*right*) VA-TSH RIA, B_0/T = 30%

Table 2. Coefficients of Variation for the Three Assays

Abbott			Clin. Assays[a]			VA TSH assay[b]		
TSH concn, milli-int. units/L	CV, %		TSH concn, milli-int. units/L	CV, %		TSH concn, milli-int. units/L	CV, %	
	Within assay[a]	Between assay[c]		Within assay	Between assay		Within assay	Between assay
2.0	4.4	16.1	4.8	6.7	12.7	1.0	23.4	41.5
6.25	7.2	15.3	17.3	4.1	19.1	2.0	16.8	24.5
22	4.0	14.8	21.3	3.3	19.2	4.0	10.7	12.5
33	3.9	13.1				8.0	15.5	23.2

[a] $n = 5$. [b] $n = 15$. [c] $n = 12$.

pared with that for the VA TSH assay, 0.3 milli-int. unit/L, may be attributable in part to this use of an enzymatically catalyzed product for detection. With the Pentawash device, three cycles of spraying with distilled water to five beads simultaneously, followed by aspiration of the bead wash, is performed at two different stages of the assay. This intensive washing of the beads, although somewhat inconvenient, is essential for obtaining a low background absorbance. The OPD solution, which is a substrate for the horseradish peroxidase, is not very stable, and will gradually yield an increasing background of absorbance at 492 nm. OPD is toxic and may be carcinogenic (*5*).

The mean TSH values for euthyroid subjects as measured with the Abbott and Clinical Assays kit were very similar. These were both significantly higher, however, than the mean obtained with the VA TSH assay (Table 1). Higher values for the Abbott kit as compared with the VA TSH assay was an unexpected result because the mean TSH values in the conventional TSH RIA have consistently fallen as the assay minimum detectable dose has declined (*2*). This process has been attributed to a greater proportion of detectable TSH values for serum samples with very low TSH, even with relatively insensitive assays, owing to failure to correct for the "serum effect" by adding TSH-free human serum to the assay calibrators (*2*). In the case of the Abbott assay, an increase in the proportion of sera from hyperthyroid individuals with detectable TSH may be attributed to its much greater assay sensitivity as compared with the VA TSH assay. The mean value for the hyperthyroid serum samples detectable in the Abbott kit was close to the minimum detectable dose of the VA TSH assay. The Abbott results for euthyroid subjects were higher than those obtained in the VA assay, probably owing to the 73% potency of the Abbott TSH standards measured relative to the 68/38 standard in the VA assay (*6*).

Table 3. Cross Reactivity[a] of Some Glycoprotein Hormones in Each of the Assays

Hormone (human)	Concn tested, int. units/L	Apparent TSH response, milli-int. units/L	Cross reactivity, % Abbott	Clin Assays	VA TSH
Lutropin	3000	12[b]	1×10^{-3}	0.2	0.42[b]
Follitropin	1000	14[b]	1×10^{-3}	0.2	0.29[b]
Choriogonadotropin	9×10^5	2	1.5×10^{-7}	<0.1	$<10^{-4}$

[a] Defined as (measured TSH value in int. units/L divided by the concentration of the cross-reacting analyte in int. units/L) × 100.
[b] Cross reactivity due to residual TSH contamination.

All assays compared in this study showed very good correlation for values in the hypothyroid range. The mean values for the samples from the hypothyroid patients measured in the Abbott kit significantly exceeded those obtained with the Clinical Assays method, but they did not differ from similar data obtained with the VA assay.

The minimum detectable dose for radioimmunometric or immunoenzymatic methods that rely on monoclonal antibodies bound to a bead are limited by the nonspecifically

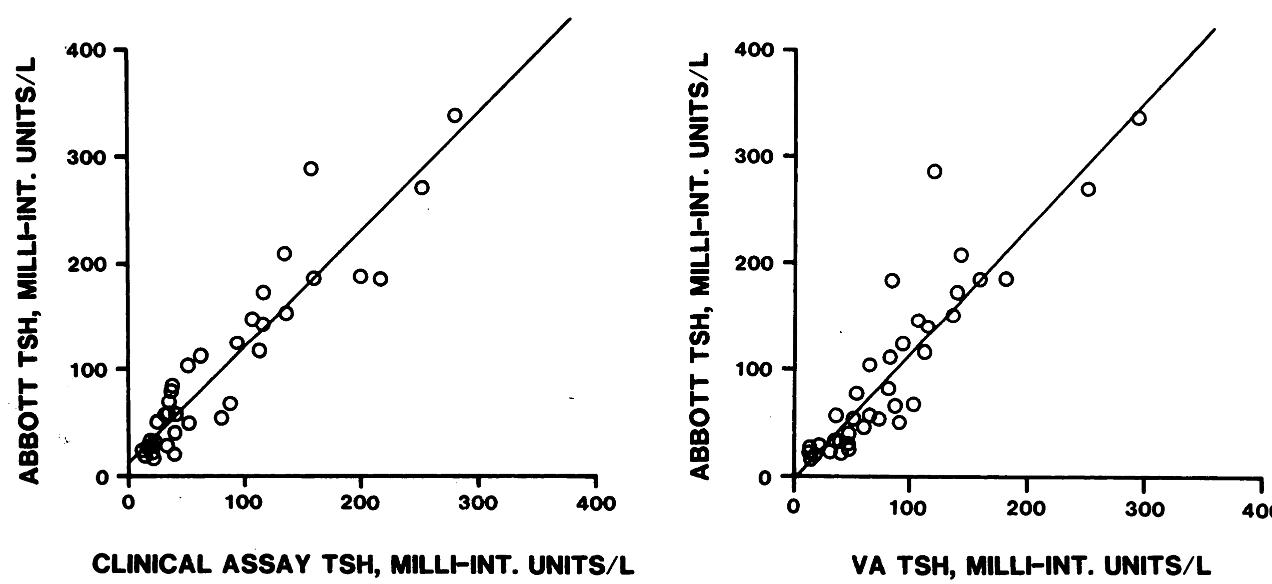

Fig. 2. Regression analysis of TSH values for 40 hypothyroid patients as measured by (*left*) the Abbott and Clinical Assays methods ($y = 1.081x + 12.28$, $r = 0.936$) and (*right*) the Abbott and VA TSH RIA methods ($y = 1.172x' - 1.57$, $r = 0.908$)

bound counts or background optical signal, respectively (see Figure 1, left panel). That for radioimmunoassays such as the Clinical Assays kit and the VA assay depend on the experimental error at zero dose divided by the antibody affinity constant (see Figure 1, middle and right panel, respectively). The apparent antibody affinity constants for polyclonal antisera may be higher than those for the constituent immunoglobulin molecules because of the occurrence of cyclic structures involving two immunoglobulin molecules with different epitope-recognition sites binding two hormone molecules simultaneously (*7*). Polyclonal RIAs, such as the Clinical Assays and VA assay, are limited by the apparent affinity constant, availability of antiserum, and burdensome isotope regulations. The new Abbott assay, on the other hand, is limited by the nonspecific signal at zero dose, which may be further minimized by advances in the detection system. Environmental concerns regarding the *o*-phenylenediamine used in the Abbott assay appear to be the only potential drawback to this new, very sensitive, nonisotopic method for measuring human serum TSH.

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CLIN. CHEM. 32/3, 514–518 (1986)

A Sensitive and Practical Immunoradiometric Assay of Thyrotropin

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We describe a two-site immunoradiometric assay for thyrotropin (TSH) in serum, based on use of two monoclonal antibodies directed against two separate antigenic determinants on the TSH molecule. One antibody is immobilized on polystyrene beads; the other is radioiodinated by a modified Chloramine T method. The detection limit of the assay is 0.02 milli-int. unit/L. The working range (CV <10%) is from 0.1 to >50 milli-int. units/L. The log mean concentration of TSH in sera collected from 100 euthyroid subjects between 08:00 and 11:00 hours was 1.9 milli-int. units/L, the range 0.4–5.4 milli-int. units/L. Values for hyperthyroid patients and thyroid-cancer patients being treated with thyroxin were much lower than those for euthyroid persons. Results by this new assay correlated excellently with those by our conventional radioimmunoassay ($r = 0.99$) and also with a sensitive immunofluorometric TSH method (Delfia™ TSH) ($r = 0.99$).

Additional Keyphrases: *monoclonal antibodies · hyperthyroidism · thyroid-cancer patients · reference values · radioimmunoassay and immunofluorometric assay compared · preparing TSH-free serum · time of day as a factor in sampling*

The limited sensitivity of most radioimmunoassays (RIAs) for thyrotropin (TSH) means that TSH concentrations are recorded as undetectable, not only in hyperthyroid patients but also in some euthyroid subjects. There are sensitive RIA methods (*1–4*), but they usually require long incubation times and are relatively labor intensive. Recently, by using immunometric procedures, it has been possible to develop very sensitive TSH assays. In these one or more monoclonal antibodies are labeled with horseradish peroxidase (*5*), ^{125}I (*6–8*), a europium chelate (*9*), or a chemiluminescent acridinium ester (*10*). Such assays are sensitive to <0.1 milli-int. unit of TSH per liter, making it possible to distinguish results for hyperthyroid subjects from those for euthyroid individuals.

We have developed a sensitive and practicable immunoradiometric TSH assay that has worked well during six months of routine use. Results by this new assay compare well with those by our old radioimmunoassay method and by a commercial immunofluorometric method.

Materials and Methods

Immunoradiometric TSH Assay

Standard TSH containing 150 milli-int. units of human pituitary TSH (code 68/38) per ampoule was obtained from the National Institute for Biological Standards and Control, London, U.K. We prepared standard solutions containing 125, 62.5, 10.4, 3.47, 1.16, 0.19, 0.095, 0.047, and 0.023 milli-int. units/L by dilution with human TSH-free serum.

TSH-free serum was initially purchased from International Laboratory Services, London, U.K., but subsequently we prepared it ourselves. To do so, we used two immunoadsorbents, produced by reacting 40 mg of monoclonal antibody (Medix Biochemica, Finland, clones 5403 or 5402) with 12.5

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Received October 7, 1985; accepted November 26, 1985.