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Sensitive Time-Resolved Immunofluorometric Assay of Thyrotropin in Serum

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We have developed a time-resolved solid-phase immunofluorometric assay for thyroropin (TSH). The assay is performed in white opaque microtitration wells which are coated with a monoclonal capture antibody. Serum TSH binds simultaneously to the solid phase and to a biotinylated monoclonal detection antibody. The degree of biotinylated antibody binding is quantitated with streptavidin conjugated to thyroglobulin which is heavily labelled with the Eu^{3+} chelator 4,7-bis [chlorosulfophenyl] -1,10-phenanthroline -2,9-dicarboxylic acid (BCPDA). The final fluorescent complex is measured on the solid phase with time-resolved fluorometry. The assay requires two incubation steps and can be completed in 5 hours. The detection limit is 0.03 milli-int. units/L. The present assay was compared with two immunoradiometric assays and gave satisfactory results.

KEY WORDS: non-isotopic immunoassay; thyroid function tests; europium chelates; solid-phase immunoassay; biotin-streptavidin; gated fluorometry.

Introduction

Radioimmunoassays for thyrotropin (TSH) have been useful for many years in the diagnosis of primary hypothyroidism in which the serum levels of TSH are elevated (1,2). These assays have generally not been sufficiently sensitive to distinguish between normal and suppressed serum levels of TSH found in hyperthyroid patients (1,2) unless very long incubation times measured in days were used (3). The advent of more sensitive two site "sandwich" assays (4,5) coupled with the use of monoclonal antibodies to increase specificity (6) has permitted the development of TSH assays which can be completed in hours. Methodologies based on radioactivity (7–10), chemiluminescence (11), enzymatic reactions (12,13) and time-resolved fluorescence (14–16) have been employed. These assays are sensitive enough to differentiate between the TSH levels found in the normal population and those associated with primary hyperthyroidism and are now being evaluated as "first-line" thyroid function tests (17–19).

Due to the problems inherent in the use and disposal of radioisotopes, methodologies using non-isotopic detection systems have gained acceptance. Recently there has been increasing interest in time-resolved fluorescence using complexes of rare earth metals and particularly europium (20–22). We have recently described a new europium chelate, 4,7-bis (chlorosulfophenyl)-1, 10-phenanthroline-2,9-dicarboxylic acid (BCPDA) which is suitable for use in time-resolved fluorometry (23). BCPDA can be used to directly label proteins for use in solid-phase fluoroimmunoassays carried out in microtitration wells. In the presence of excess europium, the fluorescence of labelled proteins bound to the bottom of the wells can be determined on the dry surface. We have reported the use of such a system in immunofluorometric assays for alpha-fetoprotein (24) and human choriogonadotropin (25) and in fluoroimmunoassays for cortisol (26,27).

In this paper we describe a sensitive assay for TSH which can be used to distinguish the suppressed, normal and elevated serum levels of TSH associated with hyperthyroid, euthyroid and hypothyroid patients, respectively.

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Materials and methods

Chemicals

The europium chelator 4,7-bis (chlorosulfophenyl)-1, 10-phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesized as described in (23). Bovine serum albumin (BSA, RIA grade), bovine globulin, thyroglobulin and streptavidin were purchased from Sigma Chemical Co., St. Louis, MO 63178. Calstan™-II standard and human choriogonadotropin standard (hCG) were from Calbiochem-Behring, San Diego, CA 92112. Affinity purified follitropin (FSH) and lutropin (LH) were purchased from Scripps Laboratories, San Diego, CA 92131. Gentamicin was purchased as a 50 mg/mL solution from Gibco Canada, Burlington, Ontario L7P 1A1. Sulfosuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin) was from Pierce Chemical Co., Rockford, IL 61105. $EuCl_3$ hexahydrate was purchased from Aldrich Chemical Co., Milwaukee, WI 53201. TSH standards in a serum matrix were purchased from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario L4X 2C8. The monoclonal antibodies used are shown in Table 1. They are available from CyberFluor, Inc. All other chemicals were from Sigma. White opaque 12-well mi-

TABLE 1
Monoclonal Antibodies Tested

Monoclonal Antibody Code	Specificity	Affinity
001	Whole TSH	2×10^{10}
002	β-TSH	3×10^{10}
003	Whole TSH	6×10^{9}
004	Whole TSH	2×10^{10}
005	Whole TSH	2×10^{9}
006	Whole TSH	2×10^{9}
007	Whole TSH	1×10^{10}
008	Whole TSH	5×10^{8}
009	Whole TSH	3×10^{10}
010	not available	5×10^{9}
011	not available	2.5×10^{8}
012	not available	not measured
013	not available	not measured
014	not available	not measured
015	β-TSH	1×10^{10}
016	β-TSH	2×10^{10}
017	Whole TSH	2×10^{10}
018	β-TSH	2×10^{10}
019	β-TSH	2.4×10^{9}
020	β-TSH	5.5×10^{8}
021	β-TSH	2×10^{10}

crotitration strips are a product of Dynatech Labs, Alexandria, VA 22314.

COMPARISON METHODS

For comparison studies we used two assays; the EchoClonal™ TSH immunoradiometric assay (IRMA) from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario L4X 2C8 and the Allégro™ HS-TSH IRMA from Nichols Institute, San Juan Capistrano, CA 92675.

INSTRUMENTATION

Time-resolved, solid-phase fluorescence measurements were performed on the Model 615 Immunoanalyzer available from CyberFluor, Inc., Toronto, Canada. This instrument is a gated fluorometer using a nitrogen laser as an excitation source and measures surface or solution fluorescence in microtitration wells. Specially designed software allows for automated data reduction by spline smoothing techniques.

Radioactivity was measured with a 1272 Clinigamma counter (LKB Wallac, Turku, Finland).

METHODS

Solutions

For antibody selection, TSH standards were prepared from Calstan™-II which contains TSH, FSH, LH and growth hormone (hGH). It was diluted in 50 mmol/L phosphate buffer at pH 7.6 containing 9 g NaCl and 5 g BSA per liter (PBS-BSA). Biotinylated antibodies were diluted in testing buffer which consists of 50 mmol/L Tris-HCl at pH 7.8 containing 9 g NaCl, 5 g BSA, 0.5 g bovine globulin. 0.1 mL polyoxyethylenesorbitan monopalmitate (Tween 40) and 0.5 g NaN_3 per liter.

In the final assay, TSH standards at concentrations of 0.3, 1.5, 5.0, 25.0 and 100.0 milli-int. units/L were prepared in a Tris buffer solution (50 mmol/L Tris-HCl at pH 7.8) containing 50 g BSA, 0.5 g NaN_3, 0.1 g thimerosal and 0.1 g gentamicin per liter. Biotinylated antibodies were diluted in Tris assay buffer (50 mmol/L Tris-HCl at pH 7.8) containing 9 g NaCl, 111.9 g KCl, 5 g BSA, 0.5 g bovine globulin, 0.5 g NaN_3 and 5.0 mL normal mouse serum per liter. The streptavidin-europium buffer was a 50 mmol/L Tris solution of pH 7.2 which contained 40 g BSA and 0.5 g NaN_3 per liter. Four mL of a 1×10^{-3} mol/L $EuCl_3$ stock solution prepared in 0.01 mol/L HCl were added to 100 mL of the solution above to complete the preparation of the streptavidin-europium buffer. The wash solution contained 9 g NaCl, 0.5 ml polyoxyethylenesorbitan monolaurate (Tween 20) and 0.5 g NaN_3 per liter.

Labelling of streptavidin

Streptavidin was directly labelled with BCPDA [SA-BCPDA] as described in (28). Alternatively, streptavidin was conjugated to thyroglobulin which had been labelled with BCPDA [SA-TG-BCPDA]. This procedure is described in detail elsewhere (29).

Biotinylation of antibodies

One mL of antibody solution (1.0 mg/mL) was dialysed overnight at 4°C against 4 L of a 9 g/L NaCl solution. To the antibody solution was added 0.4 mL of 0.5 mol/L sodium carbonate of pH 9.1 and 2 mg of NHS-LC-biotin dissolved in 100 μL dimethyl sulfoxide. The mixture was gently shaken for 1 h and dialyzed twice against 4 L of saline. The antibody was diluted to a 200 μg/mL stock solution with 50 mmol/L sodium phosphate buffer at pH 7.4 containing 1 g gelatin and 9 g NaCl per liter.

Coating of microtitration strips

The strips were coated overnight at room temperature with 100 μL per well of 0.1 mol/L $NaHCO_3$ solution containing 2 μg/mL of antibody. The strips were washed twice with wash solution and blocked for 1 h at room temperature with 300 μL per well of 0.1 mol/L $NaHCO_3$ solution containing 10 g BSA per liter. The strips could be stored with the blocking solution for one or two weeks at 4°C.

Testing of antibodies

The antibodies were tested in pairs using each as a coating or as a detection antibody. Calstan™ standards were diluted in PBS-BSA buffer to 5 and 50 milli-int. units/L of TSH. "Sandwich" assays were done with pairs of antibodies as follows: 50 μL of buffer or Calstan™ standards were pipetted into wells coated with 500 ng of coating antibody. 100 μL of testing buffer containing

500 ng of biotinylated antibody was then added. The strips were briefly shaken, incubated for 2 h at room temperature and then washed three times with wash solution. 100 μL of streptavidin-europium buffer containing 300 ng of SA-BCPDA were added to each well and incubated for 45 min at room temperature. The strips were washed three times with wash solution, dried with a stream of cool air and then read on the 615 Immunoanalyzer.

Assay procedure

Biotinylated antibody is diluted to 3 μg/mL in assay buffer (working antibody solution). This solution is stable for at least one month at 4°C. 200 μL of standards, controls and patient samples are pipetted in duplicate into the coated wells. With a repeater pipette, 50 μL of the working antibody solution are added to each well, the strips are covered and shaken for 4 h at room temperature. After washing the strips three times with wash solution, 100 μL of SA-TG-BCPDA, freshly diluted to 250 ng/mL in terms of streptavidin concentration (29) in the streptavidin europium buffer, are pipetted into the wells. The strips are covered and shaken for 30 min at room temperature. After washing three times, the strips are dried in a stream of cool air. The surface fluorescence is determined on the CyberFluor model 615 Immunoanalyzer which also has data reduction capability and constructs the calibration curve and calculates the results.

Results and discussion

Selection of antibodies

Twenty one antibodies were tested in pairs in a "sandwich" assay as described in Methods. Most antibodies were tested for either capture (coating) or detection (biotinylated). Those pairs which gave relatively low fluorescence at zero TSH concentration and high fluorescence reading at 50 milli-int. units/L TSH concentration were investigated further. In general, the antibodies with the highest affinity performed better as coating antibodies. When paired with the same capture antibody, some of the lower affinity antibodies were more effective as detection antibodies in comparison to higher affinity antibodies (data not shown).

In the initial screening studies, 11 antibodies were used for coating. Each coating antibody was paired with each one of 17 different biotinylated antibodies. Thus, the possible number of pairs tested was 176. When the fluorescence signal obtained with a certain pair was very low, we concluded that these antibodies bind to the same or to some overlapping antigenic site of TSH and are unsuitable to be used as a pair in a two-site immunoassay system. From those pairs which gave high fluorescence signal at 50 milli-int. units/L and low signal at 0 milli-int. units/L TSH (background), we finally selected a pair which gave results for patient sera that correlated best with an immunoradiometric assay. The chosen pair was code no. 002 (capture) and code no. 006 (biotinylated).

Assay optimization

The coating of strips was optimized for antibody concentration, coating pH and blocking protein. For the standard range chosen (0 to 100 milli-int. units/L TSH), the fluorescence signal increased as the amount of antibody per well was increased up to 100 ng. No further increase in signal was observed up to 500 ng/well of coating antibody. Therefore 200 ng was chosen to be optimum.

Using 200 ng/well of coating antibody, the assay was optimized for biotinylated antibody concentration. Increasing fluorescence signal was observed as the biotinylated antibody added was increased to 100 ng per well. Additional antibody did not give a higher signal. Therefore 150 ng biotinylated antibody per well was chosen to be optimum.

Calibration curves were constructed under optimum conditions by incubating the sample in the coated strips with biotinylated antibody for 1, 2, 3, 4 and 6 h. Assay sensitivity was increased by increasing the incubation time up to 4 h. Since more precise results were obtained if the strips were rapidly shaken during incubation, we chose to incubate for 4 h at room temperature by shaking on a microtitration plate shaker.

It is known that rheumatoid factor and anti-immu-

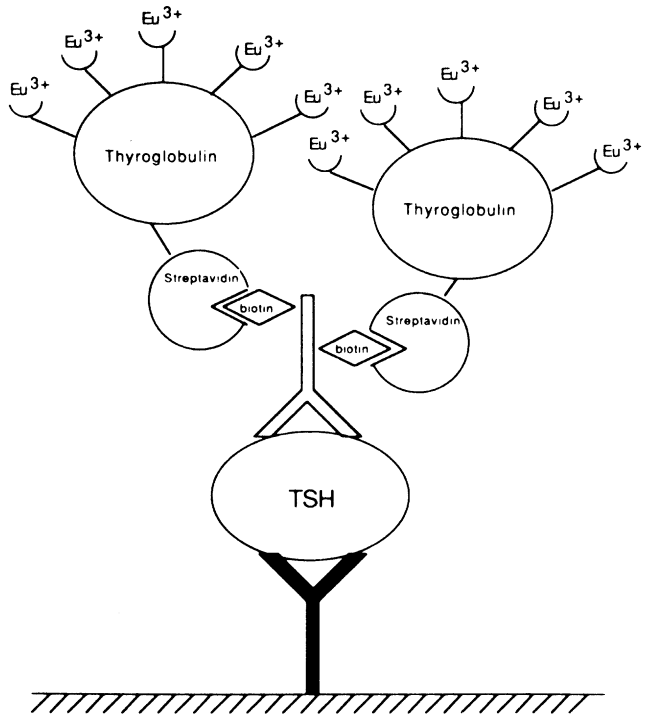

Figure 1—Schematic representation of the final complex of the TSH time-resolved solid-phase sandwich assay. The solid phase is coated with a monoclonal antibody (shown in black). The second antibody is biotinylated. Streptavidin coupled to thyroglobulin which has been labelled with BCPDA is used for detection. The figure has no quantitative meaning.

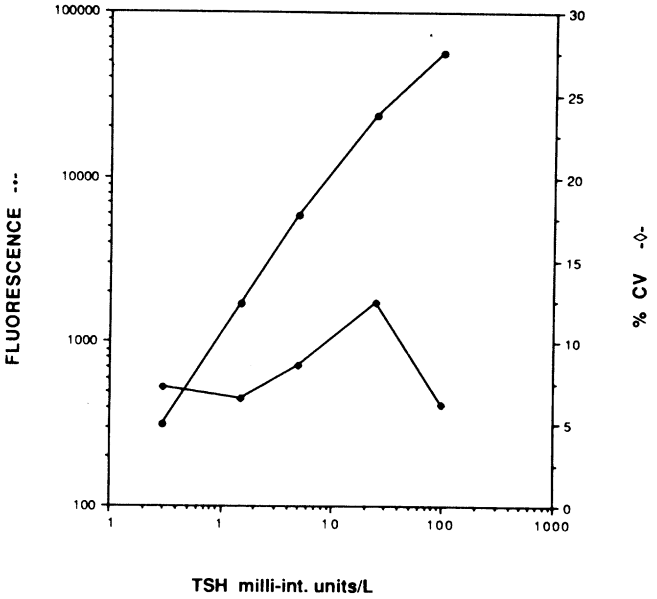

Figure 2—Calibration curve and precision profile for the TSH assay. The fluorescence of the zero standard has been subtracted from all other readings.

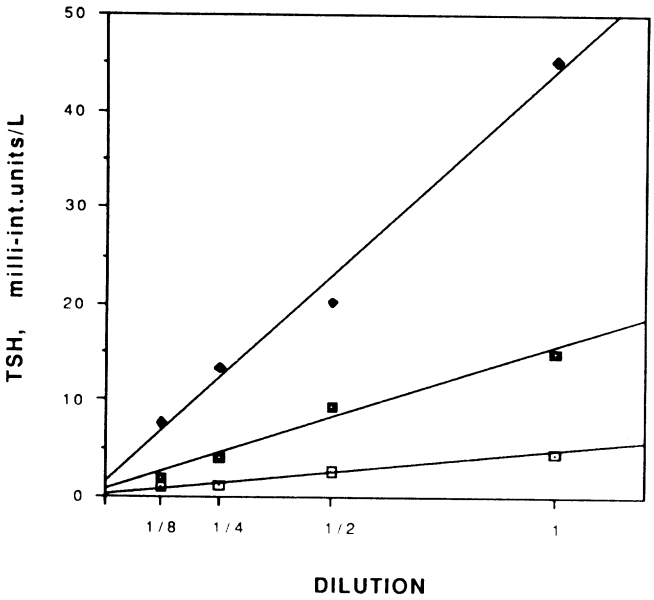

Figure 3—Linearity of results after serial dilution of three different samples with horse serum.

noglobulin antibodies can cause erroneously high results in monoclonal antibody sandwich assays by acting as a bridge between the coating and detection antibody (10,30,31). It has been reported that non-immune serum from the species in which the antibodies were raised can reverse these effects (10,30). For this reason, 5 mL normal mouse serum was incorporated per liter of assay buffer.

A schematic diagram of the final fluorescent complex in the well is shown in Figure 1.

Assay characteristics

A typical standard curve for the assay is shown in Figure 2. The data shown have been calculated by subtracting the fluorescence of the zero standard, typically 150 arbitrary fluorescence units, from the reading of all the other standards. The first standard, 0.3 milli-int. units/L, gives approximately a 3-fold higher reading than background. On a log-log plot the curve is linear up to 25 milli-int. units/L of TSH and shows a slight flattening between 25 and 100 milli-int. units/L. At very high analyte concentration, sandwich assays often exhibit a high dose "hook effect" in which the detection signal decreases with increasing analyte concentration. In this assay the curve flattens between 200 and 500 milli-int. units/L TSH. No hook effect is observed up to at least 5000 milli-int. units/L; the fluorescence is still greater than that observed for the highest standard (100 milli-int. units/L).

The detection limit of the assay, defined as the concentration corresponding to the fluorescence reading of the zero standard plus three standard deviations, is 0.03 milli-int. units/L.

Within run precision was determined by analyzing five control sera samples 12 times each. The day to day

Table 2
Precision of the TSH Assay

Sample	TSH milli-int. units/L Mean	S.D.	% CV
Within-run (N = 12)			
1	0.11	0.02	19.6
2	0.34	0.04	12.3
3	1.07	0.10	9.5
4	9.07	0.67	7.4
5	33.10	2.24	6.8
Day-to-Day (N = 10)			
1	1.20	0.11	9.3
2	9.94	0.69	6.9
3	36.13	3.60	10.0

Table 3
Recovery of TSH Added to Serum

TSH, milli-int. units/mL Initially Present	Added	Recovered	% Recovery
0.05	4.85	4.65	95.9
	14.60	10.79	73.6
0.98	4.85	5.13	85.6
	14.60	15.70	100.8
1.14	4.85	5.74	95.9
1.42	4.85	6.73	109.5
	14.60	18.06	114.0
3.51	4.85	8.65	105.8
	14.60	19.22	107.6
3.66	4.85	10.17	135.5
	14.60	20.89	118.4
3.66	4.85	9.30	116.3
16.70	4.85	20.91	86.8
	14.60	34.97	125.1

TABLE 4
Comparison of the Proposed TSH Assay to Two Immunoradiometric Assays

Comparative Method	Range milli IU/L	Slope	Intercept	r	N
Bio-Rad EchoClonal™	0–6	0.963	0.1352	0.94	173
	0–100	0.985	0.1543	0.99	183
Nichols Allégro™	0–7	0.813	0.0223	0.97	78
	0–50	1.033	−0.3285	0.99	80

precision was assessed by analyzing three serum controls 10 times over a period of two weeks. These data are shown in Table 2.

We assessed the linearity of the method by diluting three patient sera containing TSH in the range 4 to 45 milli-int. units/L with horse serum and reassaying them. The data are shown in Figure 3.

The recovery of added TSH was determined by adding a known amount of TSH to pre-analysed serum controls and patient sera. Recoveries were in the range of 74–135%. The mean value (±SD) was 105 ± 16%, where n = 14. These data are shown in Table 3.

The possible interference by hemoglobin, bilirubin (unconjugated) and triglycerides (olive oil) was also assessed. We have spiked three serum controls and two patient sera with hemoglobin (2.5 and 10 g/L), bilirubin (0.62 and 2.5 g/L) and olive oil (25 and 100 g/L) and reassayed them. The recovery of TSH was from 72 to 111% (mean 92.8%) for the hemoglobin spiked samples and 82 to 107% (mean 95.6%) for the bilirubin spiked samples. We conclude that the effect of both substances is minimal. With 100 g/L of olive oil we observed a recovery of TSH of 68 to 85% (mean 78.9%) and with 25 g/L of olive oil we recovered 83 to 100% (mean 87.5%). Severely lipemic samples might therefore be underestimated by 10–20%.

We assessed the cross reactivity of the assay with the other glycoprotein hormones. Because many preparations of lutropin (LH) and follitropin (FSH) are contaminated with traces of TSH we used affinity purified hormones. Human choriogonadotropin (hCG) and affinity purified FSH and LH were diluted with standard buffer to the appropriate concentrations. At the highest concentration of FSH and LH used (2500 int. units/L), we observed 0.05% and 0.03% cross reaction, respectively. Cross reactivity with hCG was negligible; even at the highest concentration assessed (50,000 int. units/L). At this level the fluorescence value was equivalent to 0.04 milli-int. units/L TSH (data not shown).

The accuracy of the assay was assessed by comparison with two commercial immunoradiometric assays. The results are summarized in Table 4 and Figures 4 and 5. The agreement between results is satisfactory in both cases.

A time resolved immunofluorometric assay for TSH has been previously described (14) and is commercially available from LKB Wallac, (DELFIA™). Although the proposed assay and the DELFIA™ method are both time resolved immunofluorometric assays, they differ in principle. In the DELFIA™ system, the detection antibody is labelled with Eu^{3+}. In the first step of the assay this antibody binds to TSH captured on the surface of antibody coated microtitration wells by the bound capture antibody. In the second step, a mixture of Eu^{3+} chelators is then added in excess to remove Eu^{3+} from the antibody and form a complex in solution of which the fluorescence is determined. With the DELFIA™ system care must be taken to avoid any Eu^{3+} contamination arising from skin, dust, etc. In the present assay the streptavidin is labelled with the chelator. The fluorescent complex is formed on the solid-phase by the immobilized BCPDA covalently bound to the streptavidin through the thyroglobulin carrier and excess of Eu^{3+}. Thus, it is a contamination free system. The flu-

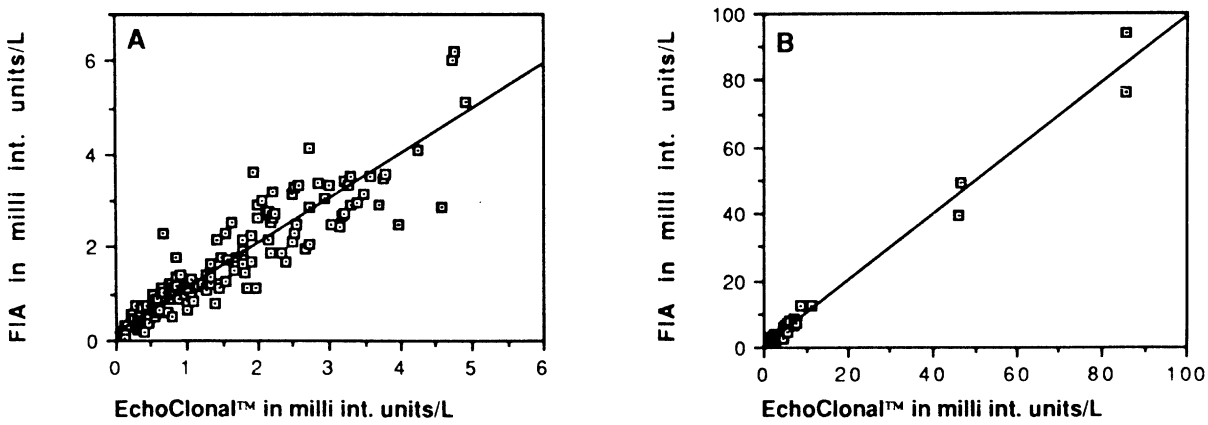

Figure 4—Comparison of the time-resolved immunofluorometric TSH assay to the Bio-Rad EchoClonal™ TSH assay plotted for the concentration range 0–6 milli int. units/L (A) and 0–100 milli int. units/L (B).

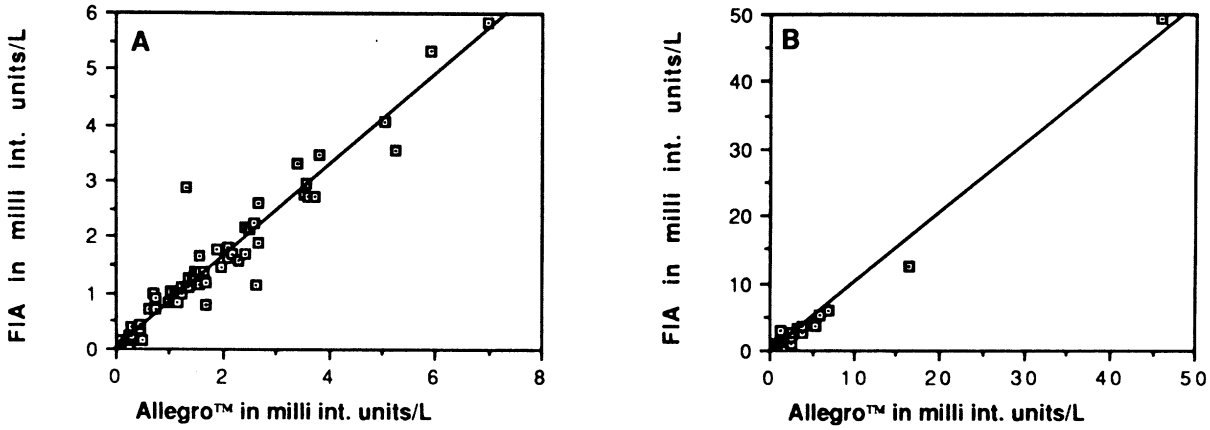

Figure 5—Comparison of the time-resolved immunofluorometric TSH assay to the Nichols Allégro™ TSH assay plotted for the concentration range 0–7 milli int. units/L (A) and 0–50 milli int. units/L (B).

orescent complex on the surface is stable for long periods of time (weeks to months).

In conclusion, we have developed a time-resolved assay for TSH which can be used to distinguish the suppressed, normal and elevated serum levels associated with thyroid disease. It requires two steps and can be completed in approximately 5 h.

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