Enzyme-Amplified Immunoassays: A New Ultrasensitive Assay of Thyrotropin Evaluated
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An enzyme-amplified immunoassay for thyrotropin has been evaluated. The lower limit of detection of the assay (mean + 3 SD of the zero standard) was 0.037 milli-int. units/L and the precision was good, giving a working range of 0.13–24.0 milli-int. units/L. The accuracy of the assay was good, as judged from analytical-recovery experiments, analysis of external quality-assessment samples and comparison with in-house assays. No significant interferences or cross reactivities were identified. Assay of a 25-μL serum sample on the microtiter plate takes 3 h. Values for healthy subjects ranged from 0.4 to 4.0 milli-int. units/L. For 32 thyrotropic patients thyrotropin concentrations were clearly suppressed and completely distinct from normal values. Patients taking triiodothyronine also showed lower concentrations. The enzyme-amplified immunoassay may thus be a suitable “front line” test for assessing thyroid function.

Additional Keyphrases: thyroid function • reference interval • thyrotoxicosis • monoclonal antibodies

Concentrations of thyrotropin (TSH) in the circulation should reflect thyroid status and pituitary function, because the secretion of TSH from the pituitary is regulated by the concentrations of thyroid hormones. Thus suppressed, low concentrations of TSH are found in thyrotoxicosis and hypopituitary patients and increased concentrations in hypothyroidism. However, many radioimmunoassays are too insensitive and imprecise to measure TSH concentrations that are below the normal range, and in clinical studies more-sensitive radioimmunoassays have had to be used, which are time consuming and require relatively large volumes of sample (1–3).

The development of immunoassays and the wider availability of monoclonal antibodies (4) has led to assays that are more sensitive and precise for many analytes of clinical interest, including TSH. In recent months many rapid and sensitive TSH assays have been described (reviewed in 5), in which both isotopic (6, 7) and non-isotopic (8, 10) labels have been used. The factor limiting sensitivity of reagent-excess assays, in contrast to RIA, is the detection limit of the label, and for non-isotopic assays gains in sensitivity have been found. But it is important to realize that the precision profile based on duplicate estimates may be more useful in assessing assay performance than an assessment of sensitivity based on 20 replicate estimations. Other factors such as reagent stability, availability of instrumentation, and safety may also determine how much use is made of these newer assays.

We report here an evaluation of a new, sensitive immunometric assay for TSH, in which enzyme amplification is used to detect bound analyte (10, 11). This system involves the use of two monoclonal antibodies to TSH, one immobilized on a microtiter plate and a second conjugated to alkaline phosphatase. After samples are incubated for 2 h with both antibodies, the plate is washed and the bound enzyme on the plate is measured kinetically by a biochemical amplification system. In the presence of substrate, NAD+ is released, which then interacts with alcohol dehydrogenase and diaphorase in a catalyzed reaction that reduces a tetrazolium salt to produce absorbance at 492 nm.

Materials and Methods
Reagents

The enzyme-amplified immunoassay kit (manufactured by IQ (Bio) Ltd., Cambridge, U.K.) includes the following: a 96-well plastic microtiter plate coated with mouse monoclonal antibody to the β subunit of TSH; a second mouse monoclonal antibody to a different epitope of intact TSH (α and β subunits) conjugated to alkaline phosphatase (EC 3.1.3.1); lyophilized substrate reagent (NADP), with buffer for reconstitution; lyophilized amplifier (alcohol dehydrogenase (EC 1.1.1.1), diaphorase (EC 1.6.4.3), p-iodonitrotetrazolium violet), with buffer for reconstitution; wash concentrate (Tris buffer with Triton surfactant); six liquid TSH standards in a bovine protein matrix, calibrated against the second IRP 80/558. Further details are given elsewhere (10, 11). Thus, in addition to the standards, 42 samples can be assayed in duplicate.

Preparations of human lutropin (12 360 int. units/mg, IRP 68/40) and follitropin (2925 int. units/mg, IRP 78/548) were kindly donated by Dr. S. Lynch, The Birmingham and Midland Hospital for Women, Birmingham, U.K. Human chorionadotropin was from Paines and Byrne Ltd., Greenford, U.K. International reference preparations for TSH (68/38 and 80/558) were from the National Institute of Biological Standards and Controls, London, U.K.

Equipment

Eight-channel pipettes (Flow Laboratories, Irvine, Scotland) were used for all additions of reagent and sample to the microtiter plates. To quantify results of the enzyme amplification assay, we used a Multiaskan MCC 340 plate reader (Flow Laboratories). A microcomputer (IQ (Bio) Ltd.) interfaced to the plate reader controls instrument function and data collection and reduction.

Procedures

Enzyme amplified immunoassay. For routine application we followed the experimental protocol provided by the manufacturer, assaying all samples/standards in duplicate. Briefly, the procedure is as follows. To each well of the microtiter plate add 75 μL of enzyme–antibody conjugate, then add 25 μL of serum or standard and incubate the plate for 2 h at 25 °C. Wash the plate four times with the wash.
buffer provided, tap it to remove wash buffer, then add 100 μL of substrate solution to each well and incubate the plate at 25 °C for exactly 20 min. Then add 200 μL of amplifier solution to each well; the Multiskan MCC 340 then automatically measures the absorbance of each well at 492 nm, at 1-min intervals for 21 min. The plate reader, being eight-channel, can read a 8 x 12 well plate in approximately 45 s. The rate of change of absorbance for each well is determined by linear regression calculation, the precision of which is improved at low absorbances by using 21 absorbance measurements. The rate of change for the standards is used to produce a calibration curve prepared by a four-parameter logistic fit. Total assay time for one plate is approximately 3 h.

**Immunoradiometric assay and radioimmunooassay for TSH.** To assess the accuracy of the kit we compared results with those obtained by an in-house IRMA, in which two mouse monoclonal antibodies are used (12). The IRMA was calibrated by use of IRP 80/558 (6). We also compared results with those by an in-house double-antibody RIA (calibrated with IRP 68/38).

Other assays. In the clinical studies, we measured total thyroxin in serum, by using an in-house RIA; free triodothyronine and free thyroxin were measured with the Amerlex-M radioimmunoassay (Amer sham International plc, Cardiff, U.K.); and TBG was measured by using an in-house immunonephelometric assay.

**Results**

**Assay Tolerance**

Figure 1 illustrates the effects of varying the duration of the first immun incubation and of the substrate incubation. Incubation temperatures between 19 and 36 °C had no detectable effect on the standard curves.

We assessed the adequacy of the washing procedure, using patients' samples with high values for alkaline phosphatase, total protein, or urea, and quality-control materials. There was no significant change in measured TSH when two, three, four, or five washes were used.

**Assay Performance**

**Lower limit of detection.** This was assessed by 26 replicate measurements of the zero diluent supplied with the kit. Calculated from the mean of the zero signal plus 3 standard deviations, the lower limit of detection was 0.037 milli-int. unit/L. We also assessed the lower limit of detection of the assay, by the method of Ekins (13), using duplicate analyses of 48 samples (standard plus patients' samples) in 10 different microtiter plates. The mean lower limit of detection was 0.013 (standard deviation 0.007) milli-int. unit/L.

**Precision.** We assessed the precision of the assay by making replicate measurements on a single plate (within batch) and by duplicate assay on 15 different plates, using quality-control materials and pooled patients' samples (Table 1). A precision profile was also obtained by using duplicate analyses (as above, ref. 13). This gave a working range for the assay (CV <10%) of 0.13–24.0 milli-int. units/L. Replicate analyses of the same sample across a plate gave no indication of assay drift when we used the assay protocol recommended by the manufacturer.

**Dose–response curve.** Two patients' samples containing high and low concentrations of TSH were mixed in various proportions and the TSH was measured. The response was linear over the range tested (0.5–15.0 milli-int. units/L, r = 0.9978, y = 986x – 0.321). Similarly, we assayed serial dilutions of patients' samples with high TSH concentrations, using the diluent provided by the manufacturer, and found a linear response: expected TSH (measured TSH): 9.57 (9.74), 4.79 (4.86), 3.83 (3.17), 1.92 (1.46) milli-int.units/L.

IRP 80/558 was added to samples to give 50, 100, and 200 milli-int. units/L and, on assay, we saw no evidence of a high-dose "hook" effect. We assayed standard curves on the same microtiter plate using the IRP 80/558 and IRP 68/38. The curves obtained were parallel, but the IRP 68/38 gave results that were 15% lower across the range (0.5–20 milli-int. units/L). Assay with the in-house IRMA gave no evidence of deterioration of IRP 68/38 during storage.

**Analytical Recovery**

Recovery of added TSH IRP 80/558 was 89% at 1 milli-int. unit/L, 85% at 5 and 10 milli-int. units/L, 87% at 20 milli-int. units/L, and 79% at 25 milli-int. units/L.

**Comparison with RIA and IRMA**

Results of the enzyme-amplified assay were compared with those by the in-house RIA, with use of samples from patients attending a thyroid clinic. The regression parameters (Deming, 14) were EAlA = (0.905 x RIA) – 0.245, r = 0.9290, n = 102. A similar comparison was made with the in-house IRMA, and the Deming line (14) was EAIA = (0.905 x IRMA) + 1.066; r = 0.922, n = 141 (Figure 2).

One sample in the method comparison showed discrepant results for TSH: IQ (Bio), 19.79 milli-int. units/L; RIA, 5.7 milli-int. units/L; IRMA, 4.5 milli-int. units/L. Initial experiments involving incubation of sample with Protein A-Sepharose or with a plain microtiter plate did not suggest that there was interference by antibodies or alkaline phosphatase. A subsequent specimen from this patient gave the following results for TSH: IQ (Bio) 16.0 and IRMA 9.0 milli-int. units/L, and when analyzed by the "Tandem-E" method (Hybritech, San Diego, CA) the TSH value was 50 milli-int.

**Table 1. Precision of Enzyme-Amplified Immunooassay for TSH**

<table>
<thead>
<tr>
<th>Samples*</th>
<th>Mean (and SD) TSH, milli-int. units/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within batch (n = 20)</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>0.245 (0.026)</td>
<td>10.7</td>
</tr>
<tr>
<td>Q2</td>
<td>1.282 (0.039)</td>
<td>3.1</td>
</tr>
<tr>
<td>P3</td>
<td>3.459 (0.136)</td>
<td>3.9</td>
</tr>
<tr>
<td>P4</td>
<td>8.347 (0.823)</td>
<td>9.8</td>
</tr>
<tr>
<td>P5</td>
<td>10.025 (0.708)</td>
<td>7.1</td>
</tr>
<tr>
<td>Q5</td>
<td>16.483 (1.490)</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Between batch (n = 15 duplicates)</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>0.268 (0.026)</td>
<td>9.8</td>
</tr>
<tr>
<td>Q2</td>
<td>1.121 (0.058)</td>
<td>5.1</td>
</tr>
<tr>
<td>P3</td>
<td>3.798 (0.271)</td>
<td>7.1</td>
</tr>
<tr>
<td>Q4</td>
<td>8.533 (0.784)</td>
<td>9.2</td>
</tr>
<tr>
<td>P5</td>
<td>9.728 (0.748)</td>
<td>7.7</td>
</tr>
<tr>
<td>Q7</td>
<td>20.889 (1.626)</td>
<td>7.8</td>
</tr>
</tbody>
</table>

*P, pooled human serum; Q, quality-control material.

**Fig. 1. Effect of immuno incubation time (right) and substrate incubation time (left) on enzyme-amplified immunoassay**

Numbers at right of curves are TSH concentrations in milli-int.units/L.
These findings will be reported in greater detail later.

External Quality Assessment

Samples sent to the laboratory as part of the U.K. External Quality Assessment Scheme for Thyroid Related Hormones were also analyzed by the enzyme-amplified assay. Good agreement was obtained with the All Laboratory Trimmed Mean (15, 16) (Table 2). This is calculated from the log-transformed results from all laboratories, with the lowest and highest 5% of the results excluded (15).

Interferences

Comparison of results from the enzyme-amplified assay and the in-house RIA for samples from patients with renal disease (n = 20), liver disease (n = 21), and myeloma (n = 5) showed no deviation from the Deming regression described above.

Similarly, a comparison of results with the in-house IRMA for samples from patients with renal disease (n = 22), liver disease (n = 21), myeloma (n = 5), high bone alkaline phosphatase activity (n = 7), and lipemia (triglycerides up to 7.0 mmol/L, n = 10) showed no deviation from the above-described Deming line. We also compared assays using samples containing high-titer autoantibodies, namely anti-mitochondrial antibody (n = 11), anti-smooth muscle (n = 4), anti-nuclear factor (n = 12), and rheumatoid factor (n = 20). No discrepant results were found.

We saw no significant interference by the following: hemoglobin (up to 5 g/L), bilirubin (up to 900 μmol/L), ethanol (up to 4 g/L), or ascorbic acid (up to 800 mg/L). No measurable cross reactivity with lutropin (up to 120 international units/L), follitropin (up to 140 international units/L), or human choriongonadotropin (300,000 international units/L).

Clinical Studies

Serum was obtained from blood sampled in the morning from 109 healthy clinically euthyroid subjects. Of these, one was excluded because of a low value for free triiodothyronine (<2.5 pmol/L, normal range 3.5–8.5 pmol/L). Samples were also obtained from 32 overtly thyrotoxic patients (total thyroxin >200 nmol/L (normal range 65–145 nmol/L) with a normal value for TBG, or a free thyroxin concentration of >60 pmol/L, normal range being 8–23 pmol/L); 20 patients with no thyroid disease but on therapy with triiodothyronine and with evidence of TSH suppression as demonstrated by subnormal total thyroxin (<10 nmol/L, and 10–26 nmol/L); and from 26 subjects older than 65 years, with no clinical evidence of thyroid disease. In addition, we analyzed serum specimens from 20 newly diagnosed hypothyroid patients (total thyroxin <65 nmol/L or free T4 <9 pmol/L, or both). An increased basal value for TSH, or exaggerated response of TSH to thyroliberin as evaluated by in-house IRMA), and from 33 hypothyroid patients on thyroid replacement therapy (basal TSH concentrations in serum by the in-house IRMA were in the range 10 to 20 milli-int. units/L).

In the normal subjects the TSH concentrations in the serum followed a non-gaussian distribution and the range was 0.4–4.0 milli-int. units/L. TSH concentrations in the elderly were similarly distributed (not significantly different according to the Mann-Whitney statistic), although one subject had a decreased value but a normal value for free triiodothyronine. Twenty-five of the 32 thyrotoxic patients had TSH concentrations of <0.04 milli-int. unit/L; the remaining seven had values well below normal, ranging from 0.04 to 0.08 milli-int. unit/L. All were significantly lower than normal (Mann-Whitney test, p < 0.001). Subjects being treated with triiodothyronine also showed suppressed concentrations of TSH (p < 0.001) (Figure 3). Of the hypothyroid patients, 13 with a total T4 of <65 nmol/L gave above-normal values for TSH, in the range of 16 to >50 milli-int. units/L (p < 0.001, Mann-Whitney test), and the remaining seven also showed increased TSH concentrations, in the range 10–32 milli-int. units/L (p < 0.001, Mann-Whitney test). In those 33 hypothyroid patients who were receiving thyroxin as replacement therapy the TSH concentrations measured with the enzyme-amplified immuneassay fell in the range 9 to 22 milli-int. units/L and were significantly (p < 0.001, Mann-Whitney test) increased. Results agreed well with the in-house IRMA results (Figure 2).

Discussion

Hitherto, use of TSH measurements as the “front-line” test in thyroid disease has been limited by the poor sensitivity and precision of conventional radioimmunoassays. This has meant that it has not been possible to distinguish the suppressed TSH concentrations of the thyrotoxic from the bottom of the normal reference interval. The recent development of immunometric assays and non-isotopic labels has
led to the introduction of more-sensitive and more-precise assays without the disadvantages attendant upon the use of isotopic labels (5).

The assay evaluated here, being an immunometric assay, would be expected to have a greater sensitivity than conventional radioimmunoassays. Reportedly (10, 11), enzyme-amplified assays for alkaline phosphatase are more sensitive than conventional p-nitrophenol-based assays. The lower limit of detection of the assay was 0.037 milli-int. unit of TSH per liter (mean + 3 standard deviations of the zero standard), which is comparable if not better than some of the newer sensitive assays (6). Most importantly, the precision of the assay is very good with a working range (defined as that for which the CV is <10%) of 0.13–24.0 milli-int. units/L. Comparison of the assay with in-house RIA and in-house IRMA for TSH showed a good correlation, though there was some evidence of bias compared with the IRMA. No significant interferences or cross reactivities were found.

One patient's sample gave discrepant results, higher values being found by the enzyme-amplified immunooassay. When measured with the Tandem-E assay, high values for TSH were also found. Initial experiments did not suggest interference by either immunoglobulins or alkaline phosphatase, and more details will be published elsewhere. Other workers (17) have described discrepant results between immunometric assays for TSH but were unable to identify the cause. Analysis of external quality-assessment material showed good agreement with the All Laboratory Trimmed Mean (15, 16), and analytical recovery of added TSH was adequate.

The reaction with the monoclonal antibodies does not go to completion during the 2-h incubation. Thus the timing of this incubation and also that with the enzyme substrate is critical for good precision. Surprisingly, neither incubation step is temperature sensitive, although incubations nevertheless should be done at controlled temperatures, away from drafts that may cause edge effects (18).

It is not clear why the enzyme activity appears to be nonlinearly related to incubation interval, or why the assay appears to be independent of incubation temperature. The kinetics of this enzyme amplification reaction may be complicated by the coupling of the enzyme label to a solid phase.

The clinical data reflect this improvement in assay performance, particularly in sensitivity and precision, as compared with the classical radioimmunoassays. In blood from healthy individuals the TSH concentration ranged from 0.4 to 4.0 milli-int. units/L, with thyrotropic persons clearly showing suppressed values, completely different from euthyroids (p < 0.001). Similarly, patients being treated with triiodothyronine showed lower concentrations of TSH, while hypothyroid patients had significantly increased (p < 0.001) concentrations of TSH.

Thus we have found the enzyme-amplified immunoassay for TSH to be very sensitive, precise, and robust. Compared with other sensitive IRMAs for TSH it has added advantages with regard to safety and the reagent stability of non-isotopic labels. The sensitivity of the assay is reflected in the clear suppression of TSH in thyrotropic patients and the improved precision profile at low TSH concentrations as compared with published (5) findings for some of the newer TSH assays. Further clinical studies of TSH values in mildly thyrotoxic and treated thyroid patients will be necessary, but our data suggest that the assay might provide a suitable "front-line" test for assessing thyroid function.

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References

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