Assessment of a Method for Measuring Serum Thyroxine by Radioimmunoassay, with Use of Polyethylene Glycol Precipitation

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We assessed the efficacy of a new thyroxine radioimmunoassay kit (Abbott) in which polyethylene glycol is used to separate bound from free hormone. Mean serum thyroxine was 88 ± 15 (±SD) μg/liter for 96 normal persons. Results for hypothyroid and hyperthyroid persons were clearly separated from those for normal individuals. Women taking oral contraceptive preparations showed variable increases in their serum thyroxine values. The coefficient of variation ranged from 1 to 3% within assay and from 5.4 to 11% among different assays. Excellent parallelism was demonstrated between thyroxine values estimated by this method and those obtained either by competitive protein binding or by a separate radioimmunoassay for the hormone.

Methods for measuring serum thyroxine (T₄) fall into two broad categories: chemical and isotopic. The most commonly used radioisotopic method is that involving competitive protein-binding assay (1). Radioimmunoassay of T₄ in unextracted serum have been increasingly used in recent years (2, 3). This report examines a radioimmunoassay method for T₄ in which polyethylene glycol is used to separate free from bound hormone.

Materials and Methods

Materials

The following materials are provided in each kit: Thyroxine standards: 0, 30, 60, 120, 240 μg/liter, in horse serum, with preservative, sodium azide, 2 g/liter.

Sheep thyroxine antiserum (cross reactivity to T₃, 0.7%) in barbital buffer (50 mmol/liter, pH 8.6) plus 7.5 g of bovine gamma-globulin, 300 mg of 8-anilino-1-naphthalene-sulfonic acid, and 1 g of thimerosal per liter.

Barbital buffer, 50 mmol/liter (pH 8.6), plus only 100 mg of thimerosal per liter.

¹²⁵I-labeled T₄ (spec. acty., 0.46 mCi/liter) in barbital buffer (50 mmol/liter) with 1 g of bovine serum and 1 g of thimerosal per liter.

Polyethylene glycol (mol wt, 6000) 180 g/liter in barbital buffer, 90 mmol/liter.

Methods

All reagents were brought to room temperature. Standard or samples to be analyzed, 25 μl, were pipetted into appropriately labeled tubes, and 0.40 ml of anti-T₄ antiserum was then dispensed into all tubes and vortex-mixed for 5 s; 0.10 ml of labeled T₄ was added to all tubes plus three total-count tubes. Each tube was placed on a vortex-type mixer for a further 5 s. The total-count tubes were capped and set aside, whereas the rack with the mixture tubes was covered with Parafilm and incubated at room temperature (22–25 °C) for 2 h. At the end of the incubation, 2 ml of PEG solution was dispensed in all tubes, except for the total count tubes and each tube vortex-mixed for 5 s. Without delay, all the tubes were then centrifuged at 3000 rpm (1500 × g) for 10 min. The supernatant fluid was promptly decanted and the lip of the tubes blotted gently. Radioactivity in the tubes was counted in a Gamma 300 counter (Beckman Instruments Inc, Fullerton, Calif.) for 1 min.

The radioactivity of the tubes was usually counted within 2 h after decanting. The percentage of bound radioactivity was calculated as a function of total radioactivity both in the case of the standards and unknown samples. Percent bound counts were plotted vs. standard T₄ concentrations on semi-log paper (Figure 1).

Serum T₄ by CPB (Tetrasorb, Abbott) and T₄ RIA charcoal, T₃ resin uptake (Triosorb, M-125, Abbott), thyroid stimulating hormone (TSH RIA, Abbott), and total T₃ (T₃ RIA, Abbott) were determined by previously established techniques.

Results

Normal range: The mean serum thyroxine for 96 euthyroid individuals was 88 ± 15 (SD) μg/liter. Women taking estrogen preparations and individuals taking drugs that affect thyroid function were excluded from this analysis. Ages ranged from 12 to 78 years and had no influence on serum T₄ concentration (r = −0.148).

Clinical efficacy: In the assessment of the T₄ PEG kit, eight women taking oral contraceptives, 11 patients with clinical features of hypothyroidism, and nine with hyperthyroidism were included (Table 1). Whereas the ingestion of oral contraceptive preparations results in an increase in the average serum T₄, we saw scatter of results from well within the normal range to beyond the 95% confidence interval. Serum thyroxine was clearly above normal in thyrotropic patients, whereas the results in hypothyroid patients were less clear-cut. The latter finding accords with the graded nature of thyroid failure (4) (Table 1).

Equivalence with other T₄ methods: Figure 2 shows the good correlation (r = 0.8866) between serum thyroxine ob-
Fig. 1. A typical standard curve for T₄ PEG RIA
The percent bound over total at zero dose of ligand (B₀) in this experiment was 78.3%. Total counts were 48 005 cpm.

Fig. 2. Correlation between T₄ values as measured by T₄ PEG-RIA and by competitive protein binding (CPB).

Table 1. Mean T₄, μg/liter ± SD, in the Various Groups Studied

<table>
<thead>
<tr>
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<th>Controls (n = 96)</th>
<th>Women on oral contraceptives (n = 8)</th>
<th>Thyrtoxics (n = 9)</th>
<th>Hypothyroids (n = 11)</th>
</tr>
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<tbody>
<tr>
<td>88 ± 15</td>
<td>111 ± 31</td>
<td>181 ± 46</td>
<td>38 ± 19</td>
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can be detected with an acceptable degree of reproducibility (CV, 20%).

Discussion

We assessed the T₄ PEG kit under routine laboratory conditions. All assays were performed by the same senior technician. The method is evidently precise and highly reproducible and can be performed with time saving over CPB and charcoal sedimentation methods. Polyethylene glycol (PEG) precipitation in the appropriate concentration can separate >95% of antigen–antibody complex instantaneously (5). The efficiency of this separation method is influenced by anions and serum protein concentration (5), but an optimal system can be easily arrived at. PEG is stable and cheap, factors that appeal to laboratories doing many assays. The test can be performed on small volumes of serum without previous extraction. Incomplete extraction of thyroxine from serum (6, 7) and problems attendant on the further treatment of the extract (7–9) have been the main drawbacks to measurement of T₄ by CPB. In contrast to our presently reported intra- and interassay variability, CV’s varying from 4.3 to 32% have been reported for CPB within the same batch as well as between batches (1, 7, 8, 10). Both the second antibody method (11) and well-optimized charcoal adsorption systems (12) achieve a similar degree of separation of free from bound hormone to that reported for PEG. However the second-antibody method requires the preparation of a suitable antiserum, and in the assay necessitates an additional period of incubation. It is also subject to nonspecific effects of plasma and anticoagulants. Optimal separation by charcoal is not without its problems, as has been recently reviewed by Hunter and Ganguli (13).

In conclusion, our investigation indicates that T₄ RIA-PEG method is a precise, highly reproducible, and economical method for the measurement of T₄ in small volumes of unextracted serum.

Kits were provided by Abbott Diagnostics Division, Abbott Laboratories, North Chicago, Ill. 60064.

References

Separation of Hydroxyproline-Containing Protein from C1q (a Subcomponent of Complement) in Serum

Carmen L. Rosano and Charles Hurwitz

Precipitation of the euglobulin fraction from serum separates C1q from another hydroxyproline-containing protein(s), which remains in the serum-minus-euglobulin fraction. The presence of C1q in the euglobulin fraction has been verified by radial immunodiffusion assay, by sensitivity to collagenase, by hydroxyproline content, and by electrodifocusing. The presence of another hydroxyproline-containing protein(s) in the serum-minus-euglobulin fraction has been verified by the same criteria. The isoelectric pH range of C1q is 6.1–7.0; the isoelectric pH range of the other hydroxyproline-containing protein(s) is 4.2–5.5.

A hydroxyproline-containing protein, called "hyproprotein," has been described, along with a method for its determination in serum, by Le Roy et al. (1). The evidence for hyproprotein was obtained by (NH₄)₂SO₄ fractionation, which revealed that 79% of the hydroxyproline-containing protein was precipitated by 40% saturated (NH₄)₂SO₄. Their fractionation of serum by Sephadex G-200 filtration showed that 55% of the hyproprotein was eluted in the α-macroglobulin and α- and β-lipoprotein range, 15% was eluted in the albumin range, and the remaining 30% trailed between the two peaks.

C1q, a subcomponent of the first component of complement, contains 4.3% hydroxyproline by weight and precipitates with the euglobulin fraction (2). In previously described procedures for isolating hyproprotein (1, 3, 4), a high-molecular-weight globulin (presumably C1q) was found to contain 50–80% of the total protein-bound hydroxyproline in serum, the rest of the protein-bound hydroxyproline being found in the non-euglobulin fraction. Incomplete precipitation of C1q from the non-euglobulin fraction might account for residual hydroxyproline in this fraction.

To test this possibility, we subjected serum to repeated precipitation in acetate buffer (20 mmol/liter, pH 5.5) according to the procedure of Lepow et al. (5), followed by extensive dialysis and electrofocusing, and found two separable protein moieties having different isoelectric pH ranges, one of which has been identified as C1q by radial immunodiffusion assay. We have also tested these two protein fractions for susceptibility to collagenase.

Materials and Methods
Separation of Hydroxyproline-Containing Protein from C1q

All separations were done on serum obtained from a pooled sample of plasma that had been defibrinated with CaCl₂. The separation was done by the method of Lepow et al. (5), with minor modifications (see Table 1). One volume of serum was added to eight volumes of acetate buffer (20 mmol/liter, pH 5.5) and allowed to stand overnight to precipitate the euglobulins. To obtain the serum-minus-euglobulin fraction, the supernatant fluid was freeze-dried, diluted to the original volume of the serum with distilled water, and then dialyzed thoroughly against isotonic saline in tris(hydroxymethyl)methylamine (50 mmol/liter, pH 7.5). To obtain the euglobulin fraction, the precipitate was washed with the acetate buffer and then dissolved in NaCl (500 mmol/liter) containing disodium ethylenediaminetetraacetate, 1 mmol/liter. Because euglobulin comprises only 4% of total serum protein, the euglobulin fraction was redissolved in 0.1 the original volume.