# A RAPID ULTRAMICRO RADIOIMMUNOASSAY FOR HUMAN THYROTROPIN

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Summary: A one day ultramicro radioimmunoassay technique for determination of thyrotropin in human plasma has been developed. The economy of the method in terms of time and reagents has been achieved without a loss of sensitivity, specificity, or precision. The results of clinical studies utilizing 20 µl of serum to measure plasma thyrotropin levels in normal subjects and patients with thyroid disease are in excellent agreement with the results achieved by longer more expensive techniques.

Several studies have demonstrated the importance of measuring plasma thyrotropin (TSH) levels in the diagnosis and management of thyroid disease (1-3). The purpose of this report is to describe a sensitive, economical, ultramicro method for the rapid determination of TSH in human plasma.

Materials and Methods: (1) 0.1 M sodium phosphate buffer (pH 7.4) in 0.85% sodium chloride containing 0.5% human serum albumin and 0.2% normal rabbit serum was used as the diluent for TSH antiserum and radioiodinated TSH.

(2) One ml of highly specific rabbit antiserum to human TSH (Calbiochem) was diluted 1:1000 with buffer. Forty µl of the antiserum (Lot No. 453039) per assay tube was sufficient to bind 40-60% of $^{125}$I-TSH in the absence of unlabeled hormone.

(3) A stock solution of TSH standard was prepared by diluting lyophilyzed Human TSH Research Standard 68/38 (Medical Research Council, Holly Hill, London) with buffer to a concentration of 12.8 mIU per ml. Working standards (0.5, 1, 2, 4, 8, 16, 32, 64, and 128 µU/ml) were prepared by further dilution of the stock solution with TSH-free human plasma obtained from subjects with severe hyperthyroidism or hypopituitarism.

(4) Purified human TSH (National Institute of Arthritis and Metabolic Disease) was radioiodinated with $^{125}I$ by a modification ( 4 ) of the method of Hunter and Greenwood ( 5 ). Pure $^{125}I$-TSH was separated from damaged protein and free $^{125}I$ by adding 125 µl of potassium iodide (10 mg/ml) in sodium phosphate buffer and 60 µl of undiluted normal rabbit serum before application of the reaction mixture to a 25 cm column of superfine Sephadex G-100 previously equilibrated with a mixture of 0.1 M phosphate buffer (pH 7.8), 0.15 M NaCl and 0.1% sodium azide. 300 µl of potassium iodide were applied after the reaction mixture and the column was eluted in 1 ml fractions with 0.1 M phosphate buffer. Damaged protein (fractions 1-14) was discarded and fractions 15-22 (containing $^{125}I$-TSH) were combined and diluted with buffer (Reagent 1) so that 0.1 ml aliquots yielded 8000 to 12000 cpm. This material, with a specific activity greater than 100 µCi/µg, could be stored at -20°C for only one week. Attempts to use the labeled hormone after this period were unsuccessful because of marked deterioration in the sensitivity and reproducibility of the assay which could not be corrected by repurification of the labeled hormone on a Sephadex column.

(5) The second antibody for separating antibody bound from free TSH was prepared by mixing 1 ml of donkey anti-rabbit serum (Wellcome) with 3 ml of 0.1 M EDTA. Titration studies demonstrated that 50 µl of this antiserum would effect complete precipitation of antibody-bound TSH in 4 hours at room temperature.

Assay Procedure: TSH standards, control serum, and unknown samples were assayed in triplicate using 400 µl disposable Microfuge tubes (Beckman). All reagents, standards, and unknowns were delivered to the assay tubes with Eppendorf or Micromedic automatic pipettes in the following sequence: (1) 20 µl of the TSH standards, various control sera, and unknown specimens were delivered to appropriate tubes; (2) just prior to use, equal volumes (5 ml) of antiserum and labeled TSH were mixed and 50 µl of the mixture was added to each tube; (3) the contents of the tubes were mixed and incubated at

room temperature for a minimum of 8 hours; (4) 50 µl of precipitating antibody was added at the end of the first incubation and after mixing the tubes were allowed to stand for an additional 4 hours at room temperature; (5) upon completion of the second incubation, 200 µl of normal saline were added and after mixing the tubes were centrifuged for 2 minutes at 10,000 x g in a Beckman Microfuge (Model 152); (6) the supernatent from each tube was aspirated and discarded using a 20 gauge disposable hypodermic needle attached to a one ml tuberculin syringe coupled to a water vacuum pump (the end of the needle was filed off so that the tip did not touch the precipitate when the hub was resting on the top of the tube); (7) the assay tubes were then transferred to 12 x 75 mm glass tubes and counted for 10 minutes or to 10,000 counts per tube in a Model 80000 LKB-Wallac automatic gamma counter; (8) data reduction was accomplished manually or by recording the counts per tube on paper punch tape (Teletype) which was analyzed by a Hewlett-Packard 9830-A calculator employing the manufacturer's "Clinical Laboratory Radioimmunoassay Software Pac, Vol. I" for logit-log transformation of the data.

Results: A typical standard curve for the radioimmunoassay of human TSH as described here is illustrated in Figure 1. A logit-log transformation of the data as suggested by Rodbard et al ( 6 ) demonstrates that the sensitivity of the assay (90% intercept) is approximately 1 µU TSH per ml of plasma with a working range up to 32 µU/ml. The precision of the assay is illustrated in Table I which shows that the coefficient of variation of replicate samples within a given assay varies from 3.7 to 7.5 per cent over a wide range of plasma TSH concentrations. The inter-assay variation of 10 per cent was established by triplicate determinations of the TSH concentration of pooled plasma in 8 consecutive assays. The mean recovery of TSH added to pooled plasma was 109 $\pm$ 13% (SD) over the range of 5 to 100 µU/ml. The validity of the method was confirmed by demonstrating parallelism between the dose response curves of serially diluted hypothyroid plasma and the TSH standard curve ( 7 ).

Plasma TSH levels in normal subjects and patients with primary hypothyroid-

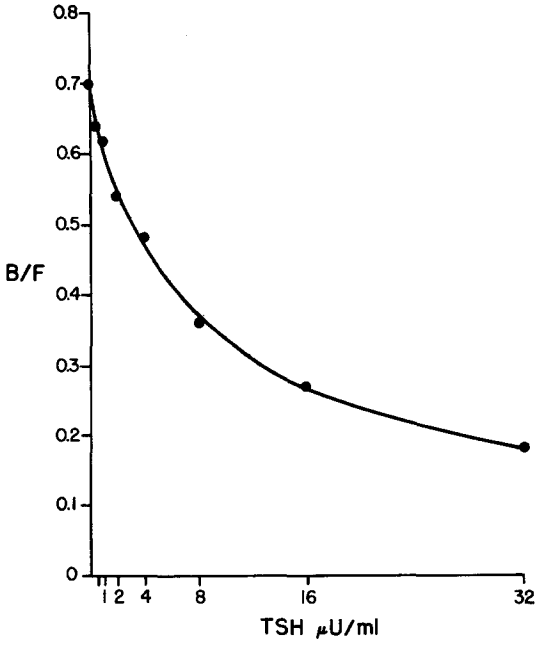


Figure 1: A typical standard curve for determination of plasma TSH using Human TSH Research Standard 68/38 diluted in TSH-free human plasma. Although standards containing 64 and 128 μU TSH per ml are included in each assay, only the steepest portion of the curve ( 1-32 μU TSH/ml) is used to determine the TSH content of unknown samples.

ism or hyperthyroidism are given in Table II. The sensitivity of the assay is such that TSH was detectable in the plasma of all but two of the normal subjects. In contrast, TSH was undetectable in plasma from 11 of the 22 patients with hyperthyroidism. Plasma TSH levels were markedly elevated in all patients with primary hypothyroidism and did not overlap the normal range.

Discussion: The radioimmunoassay technique described here provides a sensitive, reproducible, ultramicro method for rapid determination of TSH in human plasma. The small volume of unknown plasma (20 μl) needed for assay makes the technique especially useful for studies of thyroid function in infants and children. In addition, the small reagent volume saves precious materials, reduces the cost of the assay, and significantly decreases the amount of radio-

Table I: Within-assay Variance Plasma TSH

| Range μU/ml | Number Replicates | Mean μU/ml | S.D. μU/ml | Variance μU/ml | Coefficient Variation % |
|---|---|---|---|---|---|
| 2- 4 | 16 | 3.3 | 0.2 | 0.06 | 7.5 |
| 8-12 | 8 | 10.1 | 0.3 | 0.14 | 3.7 |
| 16-20 | 8 | 17.2 | 0.7 | 0.58 | 4.1 |

active waste requiring disposal. The economy of the method is also reflected by the marked reduction in time required to complete the assay. In contrast to other methods which require 3 to 5 days ( 1-4 ), the entire procedure described here can be accomplished in less than 24 hours without a significant reduction in sensitivity. Although the short incubation period may not permit the system to reach equilibrium ( 8 ), binding of the labeled hormone to antibody is satisfactory and the precision of the assay is similar to that achieved by others ( 2,4,8-10 ).

The TSH standard (68/38) used in this study appears to be similar to Human Research Standard A ( 4 ) and the range of values for plasma TSH observed in normal subjects and patients with thyroid disease do not appear to differ significantly from those reported by other investigators ( 1-3, 8, 10 ). This study also confirms previous reports that plasma TSH levels are elevated in primary myxedema and low or normal in patients with thyrotoxicosis ( 1-3, 8, 10 ).

It should be emphasized that TSH-free plasma is used as the diluent for the standard curve. This technique has been shown to decrease the binding of labeled hormone by the antiserum and to shift the standard curve to the left thereby avoiding erroneously high estimates of the TSH concentration in unknown plasma ( 4, 11-13 ).

The stability of the labeled hormone is one of the most important factors affecting the accuracy and precision of the assay ( 4,5,8,10,14 ). Although

Table II: Plasma TSH Levels (μU/ml)

| Thyroid Function | No. Subjects | Mean | S.D. | Observed Range |
|---|---|---|---|---|
| Normal | 66 | 4.4 | 1.9 | 0- 10 |
| Hypothyroidism | 15 | 136 | 51.0 | 63-244 |
| Hyperthyroidism | 22 | 2.9 | 3.3 | 0-9.3 |

high specific activity may be desirable ( 15 ), great care must be exercised to reduce iodination damage to a minimum. This goal can be partially achieved by modifying the iodination procedure as suggested by Turnbridge et al ( 4 ) but in our experience the sensitivity and reproducibility of the assay is seriously impaired if labeled hormone (stored at -20°C) is used after more than one week.

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