Simultaneous Radioimmunoassay of Thyrotropin and Thyroxine in Human Serum

M. K. Bluett,¹ E. O. Reiter,¹,² G. E. Duckett,¹ and A. W. Root¹,²

The importance of early diagnosis and treatment of congenital hypothyroidism has been well established, and several screening programs have been undertaken to detect neonates with this disorder by measurement of concentrations of thyrotropin or thyroxine in the serum. However, measurement of either hormone alone may fail to identify all affected patients. Accordingly, we have established a simultaneous double-antibody, dual-isotope radioimmunoassay for both. Sensitivity, slope, analytical recovery, and precision characteristics of the simultaneous assay do not differ from those of each assay performed separately. Values for the two analyses in the single and simultaneous assays correlate well (r = 0.951 for thyroxine, 0.983 for thyrotropin). This assay system permits determination of both hormones within 72 h after specimen collection and thus should allow more rapid evaluation, diagnosis, and treatment of infants with congenital hypothyroidism.

Additional Keyphrases: screening • inherited disorders • pediatric chemistry • hypothyroidism • thyroid status

Recent reports (1–4) suggest that the earlier congenital hypothyroidism is diagnosed and treatment begun with thyroid hormone, the better the prognosis for the affected child to have more nearly normal intellectual function. These observations have stimulated development of screening tests for detection of congenital hypothyroidism in the neonatal period (5), in which concentrations of thyrotropin (TSH)³ or of thyroxine (T₄) are measured in cord blood or in specimens obtained during the first several days of postnatal life (6–9). In most reports, two or more weeks necessarily elapsed between collection of the initial specimen and institution of appropriate treatment. We have established a radioimmunoassay (RIA) for simultaneous measurement of TSH and T₄ in serum, which permits quantitation of both hormones within 72 h after specimen collection and should enable more prompt therapy of patients with congenital primary hypothyroidism.

Materials and Methods

Materials

Table 1 lists the buffers used in these assays.

Antiserum to human TSH: We used a highly specific rabbit anti-human TSH serum (SAFP, 5-22-75) with 0.01% cross-reactivity with human chorionicadotropin, prepared and supplied by Dr. A. F. Parlow (10), at an initial dilution of 80 000-fold in Buffer A for single assays and in Buffer B for combined assays.

Antiserum to T₄: Rabbit antibody to T₄ (lot T-4-1-11/25/75) was purchased from Antibodies, Inc., Davis, Calif. 95616. The cross-reactivity of this antiserum with triiodothyronine was about 1.1%. An initial dilution of 400-fold was made in Buffer A (single assay) or Buffer B (simultaneous assay) and the diluted antiserum was stored at −20 °C.

Radioiodinated human TSH: [¹²⁵I]-TSH (Cambridge Nuclear, Billerica, Mass. 01865) was repurified before each assay by column chromatography on a 30 × 1 cm column of Sephadex G-75 that had been equilibrated and eluted with Buffer C. The initial peak of effluent radioactivity was used for assay purposes. [¹²⁵I]-TSH (human) was diluted in Buffer D so that 100 µl contained about 10 000 cpm.

Radioiodinated T₄: [¹³¹I]-T₄, obtained from American/Searle, Arlington Heights, Ill. 60005), was used without repurification. It was diluted in Buffer D so that 100 µl contained about 11 000 cpm.

Human TSH standard: Research standard A (National Institute for Biological Standards and Control, Holly Hill, London) was stored in Buffer C (plus 500 mg of gelatin per liter) in concentrated aliquots of 1 int. unit/liter and diluted in Buffer D for working standards in the RIA.

T₄ standard: A stock solution of L-thyroxine (free-acid form) from Sigma Chemical Corp., St. Louis, Mo. 63178, was prepared by dissolving 100 mg in 20 ml of 0.1 mol/liter NaOH. One milliliter of this solution was diluted with 50 ml of hormone-free serum, divided into

¹ Ed Wright Pediatric Endocrinology Research Laboratory, All Children's Hospital, 501 Sixth St. South, St. Petersburg, Fla. 33701; and the Department of Pediatrics, University of South Florida College of Medicine, Tampa, Fla. 33612.
² Reprint requests should be addressed to E. O. R., at All Children's Hospital.
³ Nonstandard abbreviations used: TSH, thyrotropin (thyroid-stimulating hormone); T₄, thyroxine; and RIA, radioimmunoassay.
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50-μl portions, and stored at −20 °C. It was further diluted with Buffer D for working standards.

**Human choriogonadotropin**: Commercially prepared material (Ayerst; Parke-Davis) was dissolved in Buffer D so that 100 μl contained 10 int. units of human choriogonadotropin and it was added to the human TSH assay mixture because of its negligible cross-reaction with the anti-human TSH serum.

8-Anilino-1-naphthalene-sulfonic acid from Sigma Chemical Co. was dissolved in the choriogonadotropin preparation to yield a concentration of 200 μg/0.1 ml. This chemical separates T₄ from its binding proteins in serum.

**Precipitating antibody**: Goat anti-rabbit γ-globulin antiserum (Antibodies, Inc.) was diluted fourfold in Buffer D.

### Radioimmunoassay Procedure

Table 2 lists the reagents for both the single and the combined assay. The reagents are added sequentially without delayed addition of any of the materials. All assays were performed in 10 × 75 mm disposable glass tubes and the final volume in each tube was 700 μl. Each tube was agitated and allowed to stand for 24 h at room temperature. Precipitating antibody (100 μl) was then added to all tubes and they were agitated and incubated overnight at 4 °C. After centrifugation (3000 rpm, 30 min, 4 °C), the supernatant fluid was decanted and the tubes were inverted and gauze swabs used to remove residual fluid. The precipitated radioactivity was sequentially counted in an automatic gamma counter at two different settings, one which discriminated for ¹²⁵I and one which discriminated for ¹³¹I. In the ¹³¹I channel, 2.2% of ¹²⁵I present was counted; in the ¹²⁵I channel, 4.0% of ¹³¹I present was counted. The data were not corrected for these overlapping counts.

Standards and unknown samples were assayed in triplicate. Control tubes containing no first antibody demonstrated nonspecific binding. Within- and between-assay CV’s for human TSH were determined in 25-μl samples of sera.

The assays were characterized by the method of Rodbard et al. (11). Log-logit transformation was used to plot the standard curve and for dose interpolation.

### Results

**T₄ (Table 3)**: The sensitivity, slope, and quality-control data for the single and combined assays do not differ. The practical sensitivity at a B/B₀ of 85% is 12 μg/liter for the single assay and 14 μg/liter for the combined assay.

The correlation (r = 0.951) between T₄ concentrations as measured in the single and combined assay systems was highly significantly (P < .001). Normal data (Table 4) reveal the discrimination between normal, hyperthyroid, and hypothyroid sera. The T₄ concentrations in cord serum were significantly higher (P < .01) than normal values in both assay systems.

**TSH (Table 5)**: The sensitivity, slope, and quality-control data for the single and combined assays for...
Discussion

The methodological principles described here are applicable to more conventional purposes in the clinical laboratory. In reported surveys (6–9, 13) affected neonates were identified by measurement of TSH or T
in cord specimens or in dried blood spots on filter paper, obtained at three to seven days postpartum. Each of the two survey methods has some disadvantage. Measurement of TSH alone does not detect infants with secondary hypothyroidism; determination of T
alone in cord serum is highly unreliable [some patients with primary hypothyroidism have had normal concentrations of T
in cord serum (6)]. Measurement of T
in dried blood spots on filter paper, obtained three to five days postpartum, might not identify infants with a failing thyroid gland who have low-normal T
concentrations.

Furthermore, in surveys utilizing T
as the screening method, TSH is measured in a subsequent assay in all specimens with low T
concentrations before recalling the infant, unless T
values are exceptionally low. This procedure has delayed diagnosis and the start of treatment for 10 days to three weeks. Thus, the use of a combined assay should be more efficient, more thorough, and permit more rapid identification and treatment of affected neonates than any method now in use. We have now validated a RIA for the simultaneous measurement of T
with a sensitivity comparable to that of standard assays and quantitation of TSH concentrations greater than 40 milliunits/liter. This TSH concentration is within the normal range in cord sera reported by Foley et al. (13), who found that neonates with congenital hypothyroidism had serum TSH values in excess of 60 milliunits/liter. Thus, the sensitivity of the TSH assay is within the acceptable range for screening purposes.

The combined assay permits rapid, accurate measurement of T
and TSH within 72 h after collection of the specimen. Use of a dual-channel gamma counter would decrease assay time still further. Although T
and TSH may be assayed separately in 8 h, such a short assay is not yet available for simultaneous measurements. In a screening program for congenital hypothyroidism, samples may be assayed twice weekly and patients with abnormal T
and TSH concentrations identified within the first six postpartum days. Therapy could then be begun as soon as repeat blood samples were obtained. If parenteral thyroxine is used to initiate treatment, the euthyroid state may be achieved within 72 h (14).

Patients with secondary hypothyroidism or thyroid-binding globulin abnormalities can also be detected by the combined T
-TSH assay, permitting more adequate evaluation and appropriate management of individual patients.

Fisher et al. (12) have recently discussed the cost/benefit ratio of large-scale screening programs. A single T
measurement currently costs about $0.60, a TSH determination about $1.50. Simultaneous assay of the two hormones would cost less than $2.00 per combined
test. The cost of finding a single infant with congenital hypothyroidism with this simultaneous assay would thus be about $10,000—far less than the estimated current cost of institutionalization of such affected patients.

This assay system is presently being validated for use in measurement of hormones eluted from dried blood specimens on filter paper.

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References