Measuring Thyroxine and Thyrotropin Simultaneously in a Dried Blood Sample on Filter Paper, to Screen for Neonatal Hypothyroidism

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We have developed a highly sensitive radioimmunoassay of thyroxine and thyrotropin for mass screening for neonatal hypothyroidism. This assay involves a single disc (3 mm diameter) of dried blood on filter paper. The minimum detectable concentrations are 15 pg/tube (10 μg/L) for thyroxine and 15 nano-int. units/tube (10 milli-int. units/L) for thyrotropin; intra- and interassay CV’s are <15% in both assays. The high sensitivity of this method is due to use of labeled thyroxine with high specific activity (3 kCi/g) and of an anti-thyrotropin serum with high affinity (Keq = 7.8 × 10-11 L/mol). With this method, 11 337 newborns were screened; a follow-up study revealed that only newborns with both high thyrotropin and low thyroxine concentrations had permanent hypothyroidism. We conclude that this method is sensitive, simple, and reliable and that the recall rate with this method is much lower than that of tests for measuring thyroxine or thyrotropin alone.

Additional Keyphrases: newborns · radioimmunoassay · reference intervals · prerequisites to permanent hypothyroidism · screening

Measurement of thyroxine (T4) or thyrotropin (TSH) in dried blood samples is very valuable in identifying neonatal hypothyroidism and thus is being widely used in programs of mass screening (1–10). It is not clear that measurement of either T4 or TSH alone suffices to detect most cases of neonatal hypothyroidism, or which one of the two tests is the more valuable. Measurement of both T4 and TSH simultaneously would be ideal if there were a simple, reliable, and inexpensive method for doing so. We describe such a method for the simultaneous radioimmunoassay of T4 and TSH and its application to screening for neonatal hypothyroidism.

Materials and Methods

Reagents

Chemicals: T4 was obtained from Sigma Chemical Co., St. Louis, MO 63172, and L-3,5-diiodothyronine from Henning Berlin GmbH. Human TSH used for immunization and for the preparation of labeled antigen was purchased from KABI Diagnostica, Stockholm, Sweden, and human TSH standard preparation (1st International Reference Preparation TSH) was kindly supplied by the National Institute for Biological Standards and Control, Holly Hill, London. Na125I and Na131I for labeling T4 and TSH were purchased from Radiochemical Centre, Amersham, England.

Anti-T4 and anti-TSH rabbit sera: Antisera to both T4 and TSH were raised in rabbits, in our laboratory. T4-bovine serum albumin conjugate, prepared by the modified technique described by Gharib et al. (11), was injected into rabbits by the multiple-site immunization procedure of Vaitukaitis et al. (12) except that pertussis vaccine was omitted. Seventy micrograms of TSH (5 int. units/mg) was injected with Freund’s complete adjuvant 10 times at four-week intervals, and blood was obtained 10 days after the last injection. The affinity constant of antibody to human TSH in the present study was 7.8 × 10-11 L/mol (Figure 1).

Labeled antigens: [125I]T4 (3 kCi/g) was prepared by iodination of L-3,5-diiodothyronine by the method of Hunter and Greenwood (13). Human TSH was iodinated with the Chloramine T method (13) to a specific activity of 70–120 Ci/g (125I-labeled TSH) or 150–250 Ci/g (131I-labeled TSH).

T4 standards: T4-free serum was prepared by incubating apparently normal pooled serum with charcoal (14); graded doses of T4 (0, 10, 20, 40, 80, and 160, and 320 μg/L) were added to make T4 standard serum.4 Erythrocytes from normal heparinized blood were washed five times with isotonic saline and once with T4-free serum, then mixed with T4 standard serum to a hematocrit of 55%. T4 standard blood was then applied to filter paper to make T4 standard dried blood. Serum T4 concentrations of T4 standard serum, measured by the “T4-RIA” kit (Eiken Immunochmical Laboratory, Tokyo), were used to establish values for T4 standard dried blood.

TSH standards: TSH-free serum was prepared by pooling normal sera containing TSH concentrations of less than 1 milli-int. unit/L; graded doses of TSH (0, 10, 20, 40, 80, and 160 milli-int. unit/L) were added to make TSH standard serum. Other procedures were the same as those used in preparing T4 standard dried blood.

Assay Procedure

We punched 3-mm diameter discs of T4 standards, TSH standards, or unknown samples from the filter paper and placed them into disposable polystyrene tubes (12 × 75 mm). To each tube we added 200 μL of antisera solution: 5 μL of anti-T4 serum, 1 μL of anti-TSH serum, 1 mL of normal rabbit serum, 500 mg of disodium ethylenediaminetetraacetate, 5 mg of 8-anilino-1-naphthalene sulfonic acid, and 1500 int. units of human choriogonadotropin in 100 mL of 50 mmol/L phosphate buffer, pH 8.0. Contents of the tubes were incubated overnight at room temperature (15–25 °C). Mean analytical recoveries of T4 and TSH from quadruplicate discs, as assessed by use of labeled T4 and TSH, were 95.8 (SD 0.6) % and 96.0 (SD 0.5) %, respectively. After 200 μL of tracer solutions (10 μCi of [123I]T4 and 10 μCi of [131I]-labeled TSH in 100 mL of buffer) was added, the samples were incubated for two days at room temperature. After incubation with 100 μL of the second antibody overnight at room temperature, the tubes were centrifuged at 2000 × g for 20 min at 4 °C, and the radioactivity of [125I] and [131I] in the precipitates was counted with a gamma counter.

4 The units for T4, throughout, are micrograms per liter of serum, because the standards are in serum.
Assay protocols for individual measurements of T₄ or TSH are summarized in Table 1.

**Screening**

In Kanagawa Prefecture, 11,337 newborns were screened with this method, blood samples being collected on the fifth through seventh postnatal days.

**Results**

**Sensitivity**

Figure 2 shows standard curves for T₄ and TSH in simultaneous and individual assays. In both T₄ and TSH assays, standard curves did not differ greatly between simultaneous and individual assays. Sensitivity, defined as the minimum dose of T₄ or TSH to decrease binding significantly by t-test, was 10 μg/L for the T₄ assay and 10 milli-int. units/L for the TSH assay.

**Precision**

Intra- and interassay precision was assessed by measuring blood samples with two different concentrations of T₄ and TSH. Intra-assay coefficients of variation (CV) for analyses of 20 samples from blood with 29 or 129 μg of T₄ per liter, or 56.3 or 129 milli-int. units of TSH per liter, were 12.6, 10.8, 8.4, and 5.7%, respectively. Interassay CV's obtained for 20 determinations of the same blood samples were 11.4, 8.1, 13.3, and 12.0%, respectively.

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**Table 1. Assay Protocols**

<table>
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<th>Individual</th>
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<tbody>
<tr>
<td><strong>Antiserum</strong></td>
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<td>Anti-T₄, Anti-TSH,</td>
</tr>
<tr>
<td><strong>mixture</strong></td>
<td>mixture, ANS, hCG*</td>
<td>mixture, ANS, hCG</td>
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<tr>
<td><strong>Pre-incubation</strong></td>
<td>overnight</td>
<td>none overnight</td>
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<tr>
<td><strong>Labeled hormone</strong></td>
<td>[¹²⁵I]T₄, [¹³¹I]-TSH,</td>
<td>[¹²⁵I]T₄, [¹²⁵I]-labeled TSH</td>
</tr>
<tr>
<td><strong>200 μL</strong></td>
<td>labeled TSH</td>
<td>TSH</td>
</tr>
<tr>
<td><strong>1st incubation</strong></td>
<td>48 h</td>
<td>24 h</td>
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<tr>
<td><strong>Second antibody</strong></td>
<td>100 μL anti-rabbit gamma-globulin goat serum; 2nd incubation, overnight; separation of bound and free antibodies by centrifugation, 2000 X g for 20 min at 4 °C</td>
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* Human chorionic gonadotropin (to abolish cross reaction with serum gonadotropins). ANS, 8-anilino-1-naphthalene sulfonic acid.

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**Fig. 1. Affinity constant of antibody to human thyrotropin**

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**Fig. 2. Standard curves for T₄ and TSH measurements**

- --- - ---: simultaneous assay of T₄ and TSH; --- O ---: individual assay of T₄ or TSH

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**Fig. 3. Correlations between T₄ or TSH concentrations as measured in serum and in discs**
Correlations between serum and disc T₄ or TSH concentrations. Thirty-one blood samples obtained from patients with various thyroid diseases were applied to filter papers; values for disc T₄ in these samples were compared with values for serum T₄ of the corresponding samples, as measured by conventional radioimmunoassay (Figure 3a). In Figure 3b are shown correlations between values for serum TSH concentrations measured by conventional assay ("HTSH" kit of Eiken Immunochemical Laboratory) and values for disc TSH in blood samples obtained from patients with mild to moderate hypothyroidism. Correlation coefficients (r) were 0.90 in T₄ and 0.94 in TSH determinations.

Correlations between simultaneous and individual measurements. Figure 4a shows T₄ concentrations in 38 neonates as determined by simultaneous and individual measurements. Values for both methods correlated well (r = 0.91). Values for TSH in simultaneous and individual assays of blood obtained from patients with hypothyroidism also correlated significantly (Figure 4b).

Screening for Neonatal Hypothyroidism

Table 2 shows the distribution of values for T₄ and TSH in 11,337 neonates, as determined by our method. T₄ concentrations averaged 103 (SD 31) µg/L, and 192 (1.7%) had concentrations of less than 40 µg/L. However, only one of the 192 had TSH exceeding 20 milli-int. units/L (140 milli-int. units/L). Fourteen newborns (0.12%) had TSH exceeding 20 milli-int. units/L, four (0.04%) exceeded 25 milli-int. units/L, one (0.01%) exceeded 30 milli-int. units/L. However, only one among these 19 patients had T₄ less than 40 µg/L (25 µg/L). A follow-up study showed that only one newborn, with 140 milli-int. units of TSH and 25 µg of T₄ per liter, had permanent hypothyroidism.

Discussion

Various methods for measuring T₄ (1-4) or TSH (4-9) in blood have been developed for mass screening for neonatal hypothyroidism. Because screening for hypothyroidism by using dried blood on filter paper is usually carried out with screening for other metabolic diseases, it is very important to reduce the size of the disc necessary for measurements of T₄ or TSH. In addition, neither theoretically nor practically can all neonates with hypothyroidism be detected by measurements of T₄ or TSH alone, and so it has been recommended that both be measured.

In previous studies, the smallest disc diameter for determining T₄ concentrations was 3.2 mm (2, 3); for measurement of TSH concentrations, two 3.2-mm diameter spots (7, 9).

In the method we present, a single 3-mm diameter disc suffices to measure concentrations of both T₄ and TSH. The assay sensitivity is similar to or more sensitive than values reported previously (2, 3, 7, 9). Although we did not make precise comparisons of this method with others, the high sensitivity of this method is probably due to using T₄ labeled with high specific activity (3 kCi/g) and an anti-T₄ serum with high affinity (association constant: 7.8 x 10¹¹ L/mol). The affinity constant of this TSH-antibody is greater than any previously reported values [4.4 x 10¹¹ L/mol, Pekary et al. (15); 1.1 x 10¹¹ L/mol, National Pituitary Agency]; 500 samples could be measured with 1 µL of this antiserum. Production of anti-T₄ serum with high affinity is very important to develop a highly sensitive immunoassay of TSH.

As shown in Table 1, this method can be used for individual measurements of T₄ or TSH if facilities for counting both isotopes simultaneously are not available or if the availability of purified TSH for frequent labeling with ¹³¹I is limited. Although four days are required to measure both T₄ and TSH, this method is simple, reliable, and inexpensive, and it seems suitable for use in mass screening programs for thyroid dys-
function. Theoretically, with this method 30% of secondary hypothyroids, or only about 1% of all hypothyroids, will be missed (16), but all cases of neonatal hypothyroidism can be detected. In fact, our screening of 11 337 newborns in this study showed only one newborn who had both increased TSH and decreased T₄ concentrations, and follow-up studies revealed that this was the only one among these newborns who had permanent hypothyroidism. Thus by simultaneous measurements of T₄ and TSH, the recall rate could be much lower than for T₄ or TSH alone.

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References