Neonatal Thyroxine Screening by Use of a Single-Tube Solid-Phase Radioimmunoassay

Ann Barbieri Bell and Larry H. Coleman

Filter paper discs saturated with dried blood can be used in the Immunotube® solid-phase thyroxine radioimmunoassay. This assay utilizes polypropylene tubes to which antibody to thyroxine is covalently bound. The filter paper standards and samples are placed in the tubes, followed by an assay buffer that contains 125I-labeled thyroxine and compounds to displace thyroxine from its binding proteins. After incubation, bound and free thyroxine are separated by aspirating or decanting the disc and buffer from the tube. The test can be used with 0.32 cm (⅜ inch) or 0.64 cm (¼ inch) discs, and gives quantitative results that correlate well with those for serum samples. The intra-assay coefficient of variation is <10%. The assay may readily be mechanized with existing disc-punching equipment, and results of its use in mass screening programs are described.

Additional Keyphrases: pediatric chemistry • screening • thyroid disease • congenital hypothyroidism • normal values for neonates

About one of every 6000 babies is born with congenital hypothyroidism, a hormone deficiency resulting in severe mental retardation (1). If the infant is treated before three months of age, however, the prognosis for achieving a normal intelligence quotient is greatly improved (2). Because the clinical symptoms before three months are often obscure, the American Thyroid Association recommends the establishment of neonatal screening programs for congenital hypothyroidism (3).

Previously reported thyroxine screening methods are hampered at the bound-free separation step. Utilizing anion-exchange resin (4), a second antibody (5), or dextran-coated charcoal (6), these systems involve at least two pipetting steps and a centrifugation, and with some methods, the thyroxine must be extracted from the filter paper before the assay. These laborious, time-consuming techniques are not readily adaptable to total automation. The following studies were done to test the adaptability of the Immunotube® T4 assay (SmithKline Instruments, Inc.) to neonatal thyroxine screening.

Material and Methods

Equipment

Gamma counter: 125I-labeled thyroxine was counted in a “Gamma System” (SmithKline Instruments, Inc.).


Materials

Thyroxine antiserum: Thyroxine antiserum was produced in rabbits according to the method of David et al. (7) with thyroxine conjugated to bovine serum albumin as antigen. Antibody immobilized on the walls of polypropylene test tubes according to the method of Barrett et al. (8) is available as the “Immunotube” T4 kit.

Isotope: 125I-labeled thyroxine from the Immunotube T4 kit was used at a concentration of 0.1 mg/liter.

Filter paper: Paper no. 903 (Schleicher & Schuell, Keene, N. H. 03431) was used in all filter paper assays.

Assay buffer: The assay buffer consisted of, per liter, 0.1 mol of 2-amino-2-hydroxymethyl-1,3-propanediol; maleic acid, 25 mmol; 0.47 mmol of 8-anilino-1-naphthalene sulfonic acid; and 0.62 mmol of sodium salicylate.

Thyroxine standards: Human serum standards were prepared from normal human serum stripped of thyroxine by charcoal adsorption. Thyroxine was then added to the standards in sufficient amounts to give final concentrations of 25, 50, 100, and 200 μg of thyroxine per liter. For use in the filter paper assay, 10 μl of standard was dried on a 0.64-cm (⅜-inch) disc or 2.5 μl was dried on a 0.32-cm (⅛-inch) disc.

Serum and blood samples: Normal serum samples from adults and corresponding anticoagulated blood samples were obtained from a local blood bank. Hematocrits of the blood samples were determined by use of a Model TE Clinette centrifuge (International Equipment Co., Needham Heights, Mass. 02194), with values read from a micro-hematocrit tube chart.

Hypothyroid samples were prepared by adding washed erythrocytes to an equal volume of hypothyroid serum samples, simulating whole blood with a hematocrit of about 50%. We prepared washed erythrocytes by washing a unit of whole blood, collected in Alsever's solution, three times with cold sodium phosphate buffer (10 mmol/liter, and containing 9 g of sodium chloride per liter).

Neonatal blood samples: Dried blood samples on 0.64-cm filter paper discs from babies three to six days of age were obtained from the State of New York Department of Health. These samples were shipped on solid CO2 within a week of specimen collection and stored at −15 °C until assayed.

Methods

Liquid serum assay: Serum sample or standard, 20 μl, was pipetted into the antibody-coated tube, followed by 2 ml of the assay buffer containing 125I-labeled thyroxine. After a 1-h incubation at room temperature, bound and free thyroxine...
Table 1. Elution of Thyroxine in Blood from Filter Paper

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total cpm on disc</th>
<th>cpm eluted</th>
<th>total eluted, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroxine, μg/liter</td>
<td>0</td>
<td>20 060</td>
<td>18 962</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>20 264</td>
<td>20 042</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>20 506</td>
<td>19 896</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>19 678</td>
<td>19 372</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19 594</td>
<td>18 866</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td></td>
<td>19 736</td>
<td>18 976</td>
</tr>
<tr>
<td>Euthyroid</td>
<td></td>
<td>19 886</td>
<td>19 604</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td></td>
<td>18 802</td>
<td>19 118</td>
</tr>
</tbody>
</table>

* Radioactivity expressed in counts per minute.

Table 2. Correlation between Liquid Serum Assay and Dried Blood Assay

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>r a</th>
<th>m b</th>
<th>b c</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.64-cm discs</td>
<td>10</td>
<td>0.90</td>
<td>1.13</td>
<td>-1.3</td>
</tr>
<tr>
<td>0.32-cm disc</td>
<td>20</td>
<td>0.91</td>
<td>0.98</td>
<td>0.3</td>
</tr>
<tr>
<td>Hypothyroid blood on discs</td>
<td>10</td>
<td>0.91</td>
<td>0.91</td>
<td>0.6</td>
</tr>
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</table>

* r = correlation coefficient.
  b m = slope.
  b c = y-intercept.

Table 3. Results of Precision Studies

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Thyroxine Mean μg/liter</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.64-cm disc</td>
<td>10</td>
<td>39</td>
<td>3.2</td>
<td>8.2</td>
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<tr>
<td></td>
<td>10</td>
<td>68</td>
<td>5.5</td>
<td>8.1</td>
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<td></td>
<td>10</td>
<td>108</td>
<td>9.9</td>
<td>9.2</td>
</tr>
<tr>
<td>0.32-cm disc</td>
<td>10</td>
<td>27</td>
<td>2.4</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>62</td>
<td>5.4</td>
<td>8.7</td>
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<td></td>
<td>10</td>
<td>100</td>
<td>9.8</td>
<td>9.8</td>
</tr>
<tr>
<td>Liquid serum</td>
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<td>62</td>
<td>2.1</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>139</td>
<td>4.3</td>
<td>3.1</td>
</tr>
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</table>

were separated by aspirating the contents of the tube. These tubes were then transferred to the gamma counter, which calculated and printed out the values for the unknown samples.

Dried blood assay: When we used blood samples, the disc containing the dried serum standard or blood sample was placed directly into the antibody tube, followed by the assay buffer containing 125-I labeled thyroxine—1 ml for a 0.64-cm disc assay or 0.5 ml for a 0.32-cm disc assay. The tubes were incubated for 2 h at 37 °C, the disc and buffer were aspirated, and the tubes then placed in the gamma counter. Because the standards were made up of serum, the values for the unknown samples obtained from the standard curve were then corrected for their respective hematocrits. For the neonatal samples, a hematocrit of 50% was assumed.

Results and Discussion

Elution of blood from filter paper: It was necessary to determine if all the thyroxine in a dried blood sample could be eluted from the filter paper and therefore be free to compete with the 125-I labeled thyroxine for binding sites on the antibody tube. Blood samples with a range of thyroxine concentrations were incubated overnight with 125-I labeled thyroxine, after which 10 μl of each was applied to a 0.64-cm filter paper disc and allowed to dry. Each disc was placed in a glass test tube, and after measuring the total counts per minute (cpm) per sample, 1 ml of assay buffer was added to each. After a 2-h incubation at 37 °C, an aliquot of buffer was counted, and from this we calculated the percent thyroxine eluted from the disc. Elution was essentially complete at all concentrations (Table 1).

Correlation between liquid serum assay and dried blood assay: To ascertain the accuracy of values obtained in the filter paper assay, we compared values for dried blood and liquid serum. Blood samples from normal adults were spotted on filter paper, allowed to dry, and 0.64-cm or 0.32-cm discs were punched from the spots and tested in the dried blood assay. Corresponding serum samples were tested in the liquid serum assay. In addition, we compared hypothyroid serum and blood samples with values ranging from 10 to 40 μg/liter by using 0.64-cm discs. The correlation in all three studies was excellent (Table 2).

Precision studies: Table 3 shows the results of replicate determinations within each of the assay systems, including the liquid serum assay. For samples of several different concentrations the disc assays show an intra-assay coefficient of variation ranging from 8.1 to 9.8%. Since the Immunotube liquid serum assay typically shows coefficients of variation in the range of 3 to 4% at both normal and abnormal concentrations, it can be assumed that the greater imprecision in the dried blood assays is due to inconsistencies in the filter paper, the spotting of the samples, or the punching of the discs.

Studies of neonatal blood samples: We obtained 350 neonatal blood samples from the State of New York Department of Health to test in the Immunotube assay. The samples were run in duplicate and divided among eight assays. Figure 1 shows the distribution of the thyroxine values for these samples. The mean value was 132 μg/liter, with a standard deviation of 35 μg/liter.
Figures 2 and 3 show the distribution of the values in these assays. An additional 27 hypothyroid neonatal samples were run in each assay system (Table 4). Using 1.5 standard deviations below the mean as a statistical cutoff point, we detected 26 of the 27 hypothyroid samples in the Immunotube assay. The samples were run singly, and the one sample that was not detected in our assay was subsequently found to be normal.

The Immunotube neonatal thyroxine assay is currently being used by the State of Washington for a statewide screening program. Blood-spot samples are taken from all infants at the time of their release from the hospital, and many pediatricians take a follow-up sample at the baby's first office visit. All samples are sent to the State Department of Social and Health Services Laboratory, where they are tested for both congenital hypothyroidism and phenylketonuria. For the Immunotube assay, the samples are run singly, with use of 0.32-cm discs; any sample with a value less than 1.5 standard deviations below that day's mean is repeated in duplicate the next day. For this statistical analysis, samples are divided into two groups, based on the age of the infant when the sample was taken. The first group of infants are those less than seven days of age, with an average of three days. To date, these samples have had a mean thyroxine value of 132 μg/liter, with a cut-off value for repeated samples of 92 μg/liter. The second group of infants are those seven days of age or older. Most of these are follow-up samples, with an average age of 3.5 weeks. The mean value for this older group is 92 μg/liter, with a cut-off value of 55 μg/liter. Samples that are suspect (about a third of those repeated in duplicate) are assayed for thyrotropin, and, if found to be abnormal, the hospital or pediatrician from whom the suspect sample was received is informed. After performing the necessary testing of the patient, the physician reports the results back to the State Laboratory.

With use of the above method, 3092 screening tests have been performed, 2950 (9.7%) of these samples being repeated in duplicate. Thyrotropin assays have been performed on 829 (2.7%) samples. To date, nine confirmed cases of primary congenital hypothyroidism have been reported back to the State Laboratory. Of these nine cases confirmed, five had initial thyroxine values, on screening, of less than 25 μg/liter. The remaining four had values of 40, 44, 60, and 70 μg/liter. All had thyrotropin values greater than 179 milliunits/liter (normal value, less than 20). In most cases, the time interval between birth and final confirmation has been less than one month.

We conclude that the Immunotube T₄ assay can be used with blood samples on filter paper, such as those now in use for screening. The assay can be mechanized when used with the Punch Indexer (Fundamental Products Co., North Hollywood, Calif. 91601), which currently is used in several laboratories for phenylketonuria screening. Discs 0.32 cm in diameter are punched out and can be simultaneously delivered to the antibody tubes and to the agar trays used in phenylketonuria testing. The "University Model" punch indexer will also deliver 0.5 ml of buffer to each tube, after dropping in the specimen disc.

Our assay provides a screening method for neonates that is accurate, versatile, and easily automated, all important features required by the State Laboratory system.
features for a large-scale screening program. The assay com-
ponents are commercially available from SmithKline In-
struments, Inc.

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References

1. Dussault, J. H., Letarte, J., Guyda, H., and Laberge, C., Thyroid
2. Klein, A., Meltzer, S., and Kenney, M. D., Improved prognosis in
congenital hypothyroidism treated before age three months. J. Ped-
iatr. 81, 912 (1972).
3. Committee of the American Thyroid Association, Recommendations
(1976).
5. Dussault, J. H., and Laberge, C., Thyroxine determination in dried
blood by radioimmunoassay: a screening method for neonatal hy-
6. Larsen, P. R., and Broeckin, D., Thyroxine immunoassay using filter
paper blood sample for screening of neonates for hypothyroidism.
7. David, B. L., and Barrett, J., Production of highly specific high titer
antisera for use in solid phase radioimmunoassay for serum thyroxine.
8. Barrett, M. J., Lemist, B., and Chow, S. Antibodies coupled to the
inside of plastic test tubes and their use in radioimmunoassay for