Immunofluorometry of Thyrotropin, from Whole-Blood Spots on Filter Paper, to Screen for Congenital Hypothyroidism

Jørgen Arends and Bent Nørgaard-Pedersen

We have evaluated a time-resolved immunofluorometric assay (IFMA) for determining thyrotropin. This "sandwich"-type system involves two monoclonal antibodies directed against different epitopes. A linear relationship between signal and thyrotropin concentration was observed up to 6000 milli-int. units/L. This procedure takes one day, vs six days with our present RIA technique, and requires only a tenth as much sample. Furthermore, intra- and interassay CVs are lower than with RIA. Assay of 19 paper-disc blood specimens from newborns identified as having congenital hypothyroidism, both by RIA and by clinical evidence, also gave positive results with IFMA. In prospective assay of 3944 specimens by both methods we identified one case of congenital hypothyroidism, which was detected by both techniques. Technical false-positive reactions, identified as such by repeated analyses, were fewer with the IFMA method than with RIA.

Additional Keyphrases: time-resolved fluorescence • pediatric chemistry • thyroid status • radioimmunoassay compared • monoclonal antibodies

Nationwide screening for congenital hypothyroidism in Denmark was started in 1977. The analytical procedure chosen was a radioimmunoassay (RIA) of thyrotropin (thyroid-stimulating hormone, TSH) eluted from blood spotted on filter paper. A worked load of about 300 such specimens a day necessitated the use of this semiautomated technique. The drawback of the RIA is its low sensitivity. Sufficient sensitivity requires a long incubation and the use of three filter-paper discs, 6 mm in diameter. The latter is a major disadvantage; little specimen is left for repeat assays or for assay of thyroxin (T4) in case of a positive result for the initial TSH assay.

Trying to overcome these problems, we evaluated a faster and more sensitive immunofluorometric assay (IFMA) and compared results with those by RIA.

Materials and Methods

Blood specimens, taken routinely by heel-puncture from all newborns on postnatal days four to six, were spotted on filter-paper (no. 818; Machery & Nagel).

In a prospective study, we analyzed 3944 specimens in parallel with both the RIA and the IFMA techniques.

In a retrospective study we retested, with the IFMA technique, 21 specimens that had been found positive for hypothyroidism by the RIA technique, of which all but two were from clinically confirmed cases of congenital hypothyroidism. These blood specimens had been kept at 4 °C for three to 23 months.

All specimens with TSH concentrations >10 milli-int. units/L are re-assayed for TSH and are also assayed for T4. If a TSH concentration >25 milli-int. units/L is confirmed and the T4 concentration is low, the case is considered to be possible congenital hypothyroidism and the patient is recalled as soon as possible to obtain a serum specimen for TSH and T4 assay. Patients with confirmed TSH concentrations between 15 and 25 milli-int. units/L and normal T4 concentration are recalled at the age of five weeks to obtain a new specimen on filter paper.

The principle of the IFMA technique has been described in a report on assay of choriongonadotropin (2). A similar technique for TSH in serum has been developed, and the reagents for it are produced and marketed by LKB-Wallac, Turku, Finland. We have modified the assay procedure described by the manufacturer, so as to be able to measure TSH in blood specimens spotted on filter paper. The modification involves substituting a filter-paper disc filled with blood for the serum specimen and preparation of TSH standards in blood spotted on filter paper. All reagents used are those supplied with the "DELFIA" serum TSH kit (code 1244-001; LKB-Wallac, Turku, Finland).

The procedure is as follows. Place a 3-mm-diameter filter-paper disc, containing about 2.9 μL of blood, in the well of a microtiter strip coated with monoclonal antibody against the beta-subunit of TSH. Elute by gently shaking for 3.5 h at room temperature with 200 μL of assay buffer containing the europium-labeled monoclonal antibody against an conformational epitope that is solely found on intact TSH and consequently allocated to the area where the two subunits associate with each other. The assay buffer has the following composition: sodium chloride (9 g/L), bovine serum albumin (5 g/L), sodium azide (0.5 g/L), bovine globulin (0.5 g/L), Tween 40 (0.1 g/L), Tris HCl (50 mmol/L), diethylenetriaminepentaacetic acid (20 μmol/L), and hydrochloric acid to give a pH of 7.75. Remove the incubation mixture with the filter-paper disc and wash the wells six times with isotonic sodium chloride solution containing 200 mg of Tween 20 polyoxyethylene (20) sorbitan monolaureate surfactant per liter. Add 200 μL of "enhancement" solution to dissociate the label from the second antibody and to form the fluorescent 2-naphthyltrifluoroacetone chelate of Eu (3), and measure the fluorescence.

The non-ionic detergent Triton X-100 in the enhancement solution keeps the chelate in a micellar solution, thus protecting the europium ion fluorescence from quenching by water molecules.

The time-resolved fluorometer we used (4) is the Arcus 1230 (LKB-Wallac). The light source, a xenon flash lamp, gives 1000 flashes per second. The fluorescence is measured in cycles of excitation, with a delay time of 400 μs and a counting period of 400 μs. The number of photons registered...
during 1000 cycles is expressed as counts per second. The
time-resolved fluorescence measurement discriminates the
short-lived (1–20 ns) background fluorescence from the long-
 lived (more than 100 ns) fluorescence produced by the
europium chelate. Results are calculated by use of a spline
function program. The detection limit of the IFMA is 5 milli-
int. units of TSH per liter in a whole-blood specimen of
about 2.9 μL contained in a disc 3 mm in diameter punched
from filter-paper blood-spots.

The whole procedure can be completed in a working day.
For both the RIA and the IFMA techniques we used
standards prepared from whole blood with a known (RIA-
measured) concentration of TSH, to which we added TSH
(1st International Reference Preparation of human TSH for
immunoassay, code 68/38, WHO, Geneva) to give final
concentrations of 5, 10, 25, 50, and 100 milli-int. units per
liter of whole blood. The hematocrit of the blood was
adjusted to 55%. We then spotted 50-μL portions of these
standards onto filter paper. In the specimen-collection kit
provided by our laboratory the same filter paper is used; it
has preprinted circles with an internal diameter of 13 mm,
within which about 50 μL of blood is absorbed and from
which the 3-mm disc is punched.

Results

A typical IFMA standard curve (Figure 1) demonstrates
the linear relationship between signal and TSH concentra-
tions, even beyond the 10 to 100 milli-int. units/L range
used in the routine assay run.

Table 1 summarizes results of analyses of the 21 retro-
spective specimens. All 19 of the patients with clinically
verified congenital hypothyroidism were correctly identified
by both methods as candidates for further laboratory and
clinical investigation. Both methods gave falsely positive
results for patient 20, who had no clinical evidence of
hypothyroidism. Patient 21 had a borderline increased
concentration of TSH. The first RIA result for TSH was 14
and by repeated assay 32 milli-int. units/L. Another serum
specimen was assayed, showing a normal TSH concentra-
tion, and there was no clinical evidence of congenital
hypothyroidism. Assays by the IFMA technique gave a TSH
value of 18 milli-int. units/L; although this value exceeds
that for normal infants (<10 milli-int. units/L), it was not
indicative of hypothyroidism (>25 milli-int. units/L).

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>First analysis</th>
<th>Repeated analysis</th>
<th>IFMA screening</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSH (mili-int. units/L)</td>
<td>TSH (mili-int. units/L)</td>
<td>TSH (mili-int. units/L)</td>
</tr>
<tr>
<td>1</td>
<td>22</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>74</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>&gt;100</td>
<td>88</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>56</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>56</td>
<td>62</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>20</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>92</td>
<td>60</td>
<td>&gt;100</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>45</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td>10–19</td>
<td>&gt;100</td>
<td>—</td>
<td>&gt;100</td>
</tr>
<tr>
<td>20</td>
<td>70</td>
<td>55</td>
<td>76</td>
</tr>
<tr>
<td>21</td>
<td>14</td>
<td>32</td>
<td>18</td>
</tr>
</tbody>
</table>

Patients 1–19 had clinically verified congenital hypothyroidism; patients 20 and 21 had normal thyroid status.

In the prospective comparison of 3944 specimens analyzed
in parallel by both the RIA and the IFMA methods, TSH was
<10 milli-int. units/L by both assays for 3868 specimens.
One specimen was positive by both assays (56 and 91 milli-
int. units/L by RIA and >100 milli-int. units/L by IFMA);
clinical investigation confirmed this to be a case of congeni-
tal hypothyroidism. Forty-seven specimens gave a positive
response in the first RIA (range 13–99 milli-int. units/L)
that was not confirmed by repeated RIA analyses, and T4
assay gave no indication of hypothyroidism. These first-
assay results are considered technical errors; all 47 speci-
mens were negative by the IFMA test. Likewise, 22 speci-
mens (0.6%) gave a positive response in the first IFMA (range
10–62 milli-int. units/L), which was not confirmed by re-
peated IFMA analysis, and all 22 were negative by RIA. Six
specimens showed TSH concentrations >10 but <25 milli-
int. units/L with both methods, and these results were
confirmed by repeated analysis. The results for TSH, paired
with the T4 concentrations, did not justify assaying another
serum specimen.

The control samples were prepared on filter paper in the
same way as the standards, with target values of 25 and 75
milli-int. units/L. We analyzed a control after every 20th
patient’s sample in the RIA and after every 12th patient’s
sample in the IFMA test run. A series of standards, in
triplicate, is included in the first positions in each assay run,
followed by patients’ samples and control in singlet. The
maximum number of patients’ samples in each run is 334.
The intra-assay mean and CV calculated for these controls,
from 12 assay series, were 25.8 milli-int. units/L and 21.8%
(n = 154) and 75.1 milli-int. units/L and 19.9% (n = 136) for
the RIA. For the IFMA the corresponding values were 28.4
milli-int. units/L and 15.3% (n = 157) and 75.1 milli-int.
units/L and 17.3% (n = 142). Interassay CVs for the RIA
were 21.9% and 29.7% for the 25 and 75 milli-int. units/L
controls; the corresponding figures for the IFMA were 13.6%
and 18.9%. The difference between the first and the last
control in each test run shows no evidence of an end-of-run
effect.

Discussion

This technique evidently is sensitive, rapid, and useful for
screening for congenital hypothyroidism. With it we could
identify all patients with verified congenital hypothyroid-
ism that we had found with the RIA we previously used.
The assay modification we used is a one-step technique with simultaneous incubation at solid-phase-bound antibody, antigen, and labeled antibody. This could give rise to a "high-dose hook effect," with decreasing signal at very high antigen concentrations (6), an effect that could lead to false negatives. However, no such effect was observed, even up to 6000 milli-int. units/L, a concentration far exceeding any to be expected in samples from patients with congenital hypothyroidism.

Concurrent falsely positive results with both methods have been seen (patient no. 21). Cases of false-positive findings in screening for congenital hypothyroidism have been described (6–11) and several causes have been pointed out. Drug and environmentally induced neonatal hypothyroidism have been reviewed (5). The most frequent causative agents are drugs used in the treatment of thyrotoxicosis and iodine ingested or used as antiseptics. Follow-up examination and maternal dietary history and drug-ingestion history are important to finding these cases.

A falsely positive reaction can also be caused by antibodies that are maternally produced and transplacentally acquired by the child. Different specificities have been found for these antibodies, including antibodies reacting with human TSH (7). Heterophilic antibodies (to IgG) that react with reagents in the immunoassay are well recognized as a potential cause of interference (8, 10). The problem is important in immunometric techniques such as IFMA where the antigen to be analyzed binds a labeled antibody to a solid-phase-bound antibody. The presence in a blood sample of a heterophilic antibody that can bind to both reagent antibodies will mimic the antigen to be analyzed. Inclusion of non-immune IgG of the same origin as the reagent antibody will overcome the problem. Addition of 10 μL of mouse serum to eluates of blood from patients 20 and 21 did not lower the measured TSH concentration, so the presence of heterophilic antibodies in these cases is improbable.

The prevalence of technical false-positive TSH values—i.e., increased (10–25 milli-int. units/L) first-test results that are not confirmed by a second analysis (<10 milli-int. units/L) based upon the original filter paper material—was 1.2% for TSH RIA but only 0.6% for IFMA.

Mean values for the control specimen agreed well between the two methods, the difference being only a few percent. For both methods the CV is somewhat better at the chosen cutoff value (25 milli-int. units/L) than for higher TSH concentrations, those highly suggestive of hypothyroidism. The IFMA technique showed a better inter- and intra-assay precision than the RIA.

We thank LKB-Wallac for a supply of the reagents.

References