Two-site Immunoenzymometric Assay for Thyrotropin in Dried Blood Samples on Filter Paper

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We describe a sensitive, simple, and rapid two-site immunoenzymometric assay for thyrotropin in dried blood samples on filter paper, for use in screening for neonatal primary hypothyroidism. In this method, two dried-blood spots of 3 mm diameter (equivalent to about 5.4 µL of blood) are incubated overnight with anti-thyrotropin–β-D-galactosidase complex in an anti-thyrotropin coated tube. Then the enzyme activity in the washed tube is determined fluorophotometrically. The range of thyrotropin measurable is 10 to 160 milli-int. units/L. Values for thyrotropin in dried blood samples determined by this method and those determined by radioimmunoassay correlated highly (r = 0.96).

**Additional Keyphrases:** screening • neonatal hypothyroidism • fluorometry • cut-off value

Because irreversible mental retardation in congenital hypothyroidism can be prevented by early treatment, several mass screening methods have been developed for early diagnosis of this disease. A sensitive radioimmunoassay of thyrotropin is one of these methods that has been widely used (1–6), but it involves radiation hazards, and the radioisotopes used have short half-lives. In 1976 we developed an enzyme immunoassay of thyrotropin that does not involve use of a radioisotope, but this method was not sufficiently sensitive to measure small quantities of this hormone in dried blood samples on filter paper for use in neonatal hypothyroid screening (7). This paper describes a new, sensitive, simple, and rapid two-site immunoenzymometric assay of thyrotropin for this purpose.

**Materials and Methods**

**Reagents**

**Chemicals:** Purified human thyrotropin was obtained from KABI Diagnostica, Stockholm, Sweden, and from Calbiochem/Behring Corp., La Jolla, CA. β-D-Galactosidase (β-D-galactosidase galactohydrolase, EC 3.2.1.23, from Escherichia coli, 5 g/L) was purchased from Boehringer Mannheim, Mannheim 31, F.R.G.; N,N'-o-phenylenedimaleimide was from Aldrich Chemical Co. Inc., Milwaukee, WI 53201; 4-methylumbelliferyl-β-D-galactopyranoside was from Sigma Chemical Co., St. Louis, MO 63178; 4-methylumbelliferone, 2-mercaptoethanolamine hydrochloride, SCAT 20-X* (a non-ionic surfactant) and Tween-20® (an anionic surfactant) were from Nakarai Chemical Co., Kyoto 604, Japan; and bovine serum albumin (Cohn Fraction V) was from Armour Pharmaceutical Co., Phoenix, AZ 85077. DEAE-Cellulose (DE-52®) was obtained from Whatman Inc., Clifton, NJ 07014; collodion bags were from Sartorius-Membranfilter GmbH, Göttingen, F.R.G.; polystyrene tubes were from Eiken Immunological Laboratory, Tokyo 114, Japan; special thick filter paper for use in mass screening was from Toyo Kagaku Sangyo Co., Tokyo 103, Japan; and Sephadex G-25, Sepharose 6B, and CNBr-activated Sepharose 6B were from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

**Rabbit anti-human thyrotropin:** Purified human thyrotropin (KABI Diagnostica) in doses of 50 to 100 micro-int. units with complete adjuvant was injected into rabbits four to eight times at three-week intervals. One month after the final injection, blood was withdrawn and serum was separated. The affinity constant of the resulting anti-thyrotropin serum was about 3.8 × 10¹⁰ L/mol. The immunoglobulin G fraction was separated from the antisera by column chromatography on DEAE-cellulose on a DE-52® column ("antiTSH-IgG"), and was further purified by affinity chromatography, as follows. The column was prepared by coupling 200 µg of thyrotropin to 2 g of CNBr-activated Sepharose 6B and equilibrating it with sodium phosphate buffer (0.1 mol/L, pH 7.5). About 22 mg of the antiTSH-IgG fraction was applied to the column and the purified anti-thyrotropin fraction was eluted with dilute (0.1 mol/L) hydrochloric acid. The eluate was dialyzed against sodium phosphate buffer (0.1 mol/L, pH 6.0) containing 1 mmol of EDTA per liter, and was concentrated by negative pressure dialysis in collection bags ("purified antiTSH-IgG").

**Tubes coated with antiTSH-IgG:** Polystyrene tubes (13 × 72 mm) were coated with antiTSH-IgG and then immunoglobulin G fraction of normal rabbit serum by the method of Yang and Kennedy (8). Polystyrene tubes were soaked in SCAT 20-X* (diluted 20-fold with de-ionized water) and washed with de-ionized water. To each tube, 200 µL of antiTSH-IgG solution (0.3 g/L in sodium carbonate buffer, 50 mmol/L pH 9.0) was added and the tubes were left overnight at 4 °C. Then the solution was removed and the tubes were rinsed with 0.15 mol/L sodium chloride solution and with the carbonate buffer. Then 300 µL of normal rabbit serum immunoglobulin G solution was introduced and the tubes were again allowed to stand overnight at 4 °C and rinsed. “Buffer A,” consisting of 10 mmol/L sodium phosphate buffer containing, per liter, 0.1 mol of NaCl, 1 mmol of MgCl₂, 1 g of NaN₃, and 1 g of bovine serum albumin, was then introduced and the tubes were stored at 4 °C.

**AntiTSH-IgG–β-D-galactosidase complex:** The antiTSH-IgG–β-D-galactosidase complex was prepared by modifications of the method of Ishikawa and Kato (9). One milliliter of purified antiTSH-IgG solution (100 mg/L) was incubated with 100 µL of 0.1 mol/L 2-mercaptoethanolamine hydrochloride for 90 min at 37 °C, under nitrogen. It was applied to a 1 × 25 cm Sephadex G-25 column. The pooled effluent from 8.0 to 10.3 mL was added dropwise, with mixing,
to 2.57 mL of a saturated solution of \( N,N'-o \)-phenylenedimaleimide in sodium acetate buffer (20 mmol/L, pH 5.0), and this mixture was promptly applied to a 1 x 45 cm Sephadex G-25 column. The absorbance of the effluent at 280 nm was monitored and peaks were detected. Fractions corresponding to the first peak (5 mL) were pooled, and adjusted to pH 6.5 with sodium phosphate buffer (0.25 mol/L, pH 7.5). To the concentrated solution, 0.4 \( \mu \)mol of \( \beta \)-D-galactosidase was added and the mixture was incubated for 20 to 40 h at 4 °C. The mixture was applied to a 1.5 x 40 cm column of Sepharose 6B, equilibrated with Buffer A, and the column was eluted with the same buffer. Two peaks of enzyme activity and three peaks of antibody activity were observed. The pooled fraction with the highest association constant, usually the second peak of antibody activity, was used. When stored at 4 °C, the complex could be used for at least six months.

**Standard thyrotropin discs**: Heparinized pooled blood from normal subjects whose serum concentration of thyrotropin was <1 milli-int. unit/L was separated into plasma and packed cells. Various quantities of standard thyrotropin (Calbiochem) were dissolved in the plasma and mixed with the packed cells, the mixtures being adjusted to the original volume of the blood. Then samples of 30 \( \mu \)L of the mixtures were spotted on the special thick filter paper used for production in mass screening. The blood samples formed dried spots of about 10 mm diameter, and discs 3 mm in diameter were cut from the spots. Such a disc contained about 2.7 \( \mu \)L of blood or 1.4 \( \mu \)L of serum, assuming the hematocrit to be 50%.

**Radioimmunoassay of thyrotropin**: A commercial assay kit (Eiken Cretin-kit®) for two-step double antibody radioimmunoassay of thyrotropin in dried blood samples was supplied by Eiken Immunochemical Laboratory, Tokyo 114, Japan. Thyrotropin in two dried 3-mm blood discs was measured with the kit and the standard thyrotropin discs described above.

**Specimens**: Dried blood samples were obtained from 1000 newborn babies in the general population and from 30 cases of primary hypothyroidism. The latter had various thyrotropin concentrations and were obtained before and during treatment with thyroid hormones.

**Assay Procedures**

Reagents were diluted with Buffer A. To each antiTSH–IgG coated tube, two 3-mm dried-blood discs (equivalent to about 5.4 \( \mu \)L of blood) obtained from standard or test samples and 150 \( \mu \)L of a 1 g/L solution of normal rabbit serum immunoglobulin G were added and the mixture was shaken gently for 10 min. Then 100 \( \mu \)L of diluted antiTSH–IgG–\( \beta \)-D-galactosidase complex was added and the mixtures were shaken gently. The mixtures were allowed to stand for 4 h at 37 °C and then for 16–20 h at 4 °C. The contents of the tube were discarded, 3 mL of "washing solution," consisting of 10 mmol/L phosphate buffer containing, per liter, 0.15 mol NaCl and 1 g of Tween 20® (pH 7.6), was introduced and the tube was allowed to stand for 10 min. The solution was discarded and the tube was washed twice with 3 mL of the washing solution and then once with 3 mL of Buffer A. To the washed tube, 250 \( \mu \)L of 4-methylumbelliferol–\( \beta \)-D-galactopyranoside in Buffer A was added as substrate. After incubation at 37 °C for 40 min, 2.5 mL of glycine buffer (0.1 mol/L, pH 10.3) was added to stop the enzyme reaction. Fluorescence was measured at 450 nm with excitation at 350 nm, with a solution of 4-methylumbelliferone in glycine buffer as standard.

**Results**

A typical standard curve obtained with use of data from standard thyrotropin discs is shown in Figure 1. Thyrotropin at concentrations of 10 to 160 milli-int. units/L of blood (equivalent to about 20 to 320 milli-int. units/L of serum) could be measured with two dried blood discs of 3 mm diameter. The minimum detectable dose was estimated as 6 milli-int. units/L of blood (equivalent to about 0.03 micro-int. units/assay tube) from the point where the 95% confidence limit of the response at zero dose intersects the standard curve (10 replicates).

As shown in Table 1, the within-assay coefficients of variation (CV) calculated from values on three samples ranged from 10.6 to 13.0% and the mean between-assay CV ranged from 11.6 to 18.3%.

The thyrotropin concentrations in 30 dried-blood samples were determined by both the present assay and radioimmunoassay. As shown in Figure 2, there was a good correlation between the values for thyrotropin determined by the two methods \((r = 0.96)\).

In preliminary screening tests using this method, one case of congenital hypothyroidism (serum thyrotropin >320 milli-int. units/L) was detected among 1000 newborn infants in the general population (the cut-off point was the upper 4 percentile). Other infants were diagnosed as normal by both the present method and radioimmunoassay.

**Table 1. Precision for Blood-spot Thyrotropin Assays**

<table>
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<tr>
<th>Samples</th>
<th>n</th>
<th>Mean milli-int. units/L</th>
<th>SD</th>
<th>CV, %</th>
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<tr>
<td><strong>Within assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>21.7</td>
<td>2.3</td>
<td>10.6</td>
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<tr>
<td>B</td>
<td>6</td>
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<tr>
<td>C</td>
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<td>76.0</td>
<td>9.6</td>
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<td></td>
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<td></td>
</tr>
<tr>
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<tr>
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<tr>
<td>F</td>
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<td>9.2</td>
<td>12.2</td>
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</table>
Discussion

Recently, several mass-screening programs for detecting neonatal hypothyroidism have been organized in which thyrotropin or thyroxine was measured by radioimmunoassay in dried-blood samples on filter paper collected to screen for other metabolic disorders (1–6). Since 1975, we have screened 148,400 babies in the general population by the method of paired thyrotropin assay using radioimmunoassay (2, 3) and have detected 25 cases of congenital hypothyroidism and eight cases of transient infantile hyperthyrotropinemia. However, the numbers of samples taken in these types of programs are so large that a non-isotopic assay would be preferable.

The enzyme immunoassay of thyrotropin that we developed in 1976 does not involve use of a radioisotope, but it is too insensitive (7). In the present method, however, the lower limit of detection is 0.03 micro-int. units per assay tube, which is significantly lower than that in our previous method (1.0 micro-int. units/assay tube) (7) or in the method of Albert (0.3 micro-int. units/assay tube) (10). The sensitivity is comparable with that of radioimmunoassay and of another sensitive double-antibody enzyme immunoassay, recently developed by Kato et al. (11) and Naruse (12) for detecting thyrotropin in dried-blood samples. The CVs of the proposed method (within assay, 10.6–13.0%; between assay, 11.6–18.3%) compare with those obtained by radioimmunoassay as follows: within-assay, 3.4–20.7% (2), 6.09–9.54% (6), 6–42% (13), 5.7–8.4% (15); between-assay, 6.73–22.89% (6), 12–38% (13), 11.8% (14), 12.0–13.3% (15). Thus, the cut-off point was not fixed; rather, the upper four percentile was selected each time in the assays.

The reliability of the present method is shown by the good correlation found between thyrotropin values determined by this method and radioimmunoassay and by the fact that one case of congenital hypothyroidism was detected by this method in preliminary mass screening tests on neonates in the general population.

The advantages of the proposed method are as follows: 1) It does not involve radiation hazards; all procedures can be done in a routine laboratory. 2) The method is sensitive; as little as 10 milli-int. units of thyrotropin per liter of blood can be detected in dried-blood samples on filter paper (two 3-mm spots). 3) The method is simple, only four pipetting, four washing, and one fluorometric procedure being involved in use of a single tube. 4) The method is rapid; all procedures can be done in two days. 5) The method is inexpensive with respect to thyrotropin; thyrotropin, which is expensive, is not used, but instead a large amount of easily prepared antiserum is used for coupling with enzyme. The enzyme–anti-thyrotropin complex is stable for at least six months. We are now attempting to develop a fully automated system for use in mass screening for neonatal hypothyroidism.

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References