Enzyme Immunoassay of Free Thyroxin in Dried Blood Samples on Filter Paper

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We describe a double-antibody enzyme immunoassay for determination of free thyroxin (FT₄) in dried blood samples on filter paper, with use of a T₄-β-D-galactosidase complex. The measurable range of FT₄ concentration in two 3-mm blood discs, each of which contained about 2.7 μL of blood, was 1.9 to 93 ng/L, as determined by comparison with concentrations of FT₄ in known serum standards. FT₄ in blood samples dried on filter paper was stable for at least four weeks when kept dry at −20 °C, room temperature, or 37 °C. The mean coefficients of variation were 7.6% (within assay) and 6.4% (between assays). Results for FT₄ by this method correlated well with those for serum determined by radioimmunoassay (r = 0.98). The proposed method can be used to differentiate persons with hyper- and hypothyroidism from normal subjects and those with abnormal concentrations of thyroxin-binding globulin. The procedure seems suited for screening studies.

Additional Keyphrases: thyroid status • newborns • screening

Mass screening for hypothyroidism in newborns is done throughout the world, because the irreversible mental retardation caused by this disorder can be prevented by early treatment (1). Moreover, the hypothalamic–pituitary–thyroid axis in premature babies has been extensively investigated. Among the methods developed for these purposes are measurements of total thyroxin (T₄) (2) and thyrotropin concentrations in dried blood samples on filter paper (2–7).
Measurement of total T₄ is useful for detecting primary, secondary, and tertiary hypothyroidism, but gives false-positive results for subjects with abnormal concentrations of thyroxin binding globulin (TBG), who do not need to be treated. Hence, we developed a radioimmunoassay method for measuring free thyroxin (FT₄) in blood samples dried on filter paper (8). Because the labeled T₄ analog used in this method is bound to albumin, however, the measured FT₄ values are influenced by the concentration of albumin in serum (9). Moreover, the large scale of screening programs makes a nonisotopic method preferable. Here we describe an enzyme immunoassay for measuring FT₄ in blood samples dried on filter paper. Results are un influenced by the concentrations of TBG and albumin.

Materials and Methods

Materials

*Chemicals:* 4-(Maleimidomethyl)cyclohexane-1-carboxylic acid succinimide ester was from Ziehen Chemical Co., Ltd., Tokyo, Japan; L-T₄ (free acid), polyoxyethylene (20) sorbitan monolaurate (Tween 20®) and o-nitrophenoxy-β-D-galactopyranoside were from Sigma Chemical Co., St. Louis, MO 63178; β-D-galactosidase (EC 3.2.1.23, from Escherichia coli; 5 g/L) was from Boehringer-Mannheim, Mannheim 31, F.R.G.; "Biogel A5m" was from Bio-Rad Laboratories, Richmond, CA 94804; rabbit antiserum to T₄; goat antiserum to rabbit immunoglobulin G, and normal rabbit serum were from Eiken Immunochemical Co., Ltd., Tokyo 114, Japan; the Amerlex® FT₄ RIA kit was from Amersham International Ltd., Amersham, Bucks., U.K.; and special thick filter paper (0.7 mm in thickness) prepared for mass screening was from Toylo Kagaku Sangyo Co., Tokyo 103, Japan.

*Immunoglobulin G:* IgG fractions were prepared from antisera and normal rabbit serum by fractionation with sodium sulfate and then passed through a column of diethylaminoethanol cellulose (10).

*T₄-galactosidase conjugate:* The T₄-galactosidase conjugate was prepared as described in detail previously (11). The T₄ was mixed with 4-(maleimidomethyl)cyclohexane-1-carboxylic acid succinimide ester, and this mixture was added to β-D-galactosidase solution and applied to a 1.5 × 45 cm column of Biogel A-5m. Fractions of the effluent containing the enzyme activity were pooled and stored at 4°C.

*Standard FT₄ in serum:* Serum containing no FT₄ ("0 ng/L") was prepared by adding activated charcoal to normal human serum and filtering (12). No FT₄ was detectable in the filtrate with the Amerlex FT₄ RIA kit. For calibration, we dissolved various quantities of T₄ in the "0 ng/L" serum and determined the concentrations of FT₄ by equilibrium dialysis (13). Heparinized pooled blood from normal subjects was centrifuged (1000 × g, 10 min) and the packed cells were washed four times with phosphate-buffered isotonic saline (0.15 mol of sodium chloride in 1 L of 10 mmol/L sodium phosphate buffer, pH 7.0). The washed packed cells were then mixed with an equal volume of the serum-based standard FT₄. Thirty microliters of the mixture, spotted on the special thick filter paper and allowed to dry at room temperature, formed a spot about 9 mm in diameter. FT₄ concentrations in the dried blood samples were expressed as equivalent to the concentrations of FT₄ in the mixture of standard FT₄ serum added to the packed cells.

Procedures

*EIA for FT₄ in dried blood samples:* The reagents were diluted with diluent: phosphate buffer (0.1 mol/L, pH 7.0) containing, per liter, 1 mmol each of MgCl₂ and mercaptethanol and 1 g of gelatin. Two 3-mm-diameter blood discs, each of which contained about 2.7 μL of blood, were punched out from standard or test blood spots on filter paper. We soaked the discs in 100 μL of anti-T₄ IgG solution (1.4 μg/L) for 30 min at room temperature (about 25°C). We then added 50 μL of solution of T₄-galactosidase conjugate (diluted 100-fold) and incubated for 5 h at room temperature. After adding 10 μL of normal rabbit IgG (0.4 g/L) and 50 μL of anti-rabbit IgG goat IgG (3.4 g/L), we incubated the mixtures for 20 h at room temperature, added 2 mL of washing solution (TWEEN 20, 1 g/L, in phosphate-buffered saline), and centrifuged the mixtures (1000 × g, 30 min). The resulting precipitates were washed twice with 2 mL of washing solution and suspended in 1 mL of a 1 g/L solution of o-nitrophenyl-β-D-galactopyranoside containing 50 mL of methanol per liter. After incubating the suspensions for 2 h at 37°C, we mixed them with 1 mL of 30 g/L sodium carbonate solution and measured the absorbance at 405 nm.

*RIA for FT₄ in serum:* We also measured serum FT₄ concentrations with an Amerlex® FT₄ RIA kit.

Subjects

Venous blood was obtained from 41 patients: seven cases of primary hypothyroidism (four adults, three neonates), two adults cases of secondary hypothyroidism due to congenital isolated thyrotropin deficiency, 12 adult cases of hyperthyroidism, and 20 cases with low TBG (eight adults and 12 neonates). Samples were also collected from 13 normal pregnant women (ages 25 to 29 years, in their 25th to 36th weeks of pregnancy) and 56 normal subjects (38 adults, ages 22 to 52 years, and 18 neonates, ages three to four weeks). Serum and dried blood spots were prepared and kept at −20°C, except for those used to study the stability of FT₄ in blood spots on filter paper.

Results

*Calibration curve:* Figure 1 shows a typical calibration curve. The minimum detectable concentration was calculat-
ed to be 1.9 ng/L of equivalent serum (5.1 fg per assay tube) from the point where the 95% confidence limit of the response at zero dose intersected the calibration curve, in six replicate determinations of FT₄ standard blood spots. We could measure FT₄ at concentrations of 1.9 to 93 ng/L.

Reproducibility: Blood, sampled from two normal subjects every week for four weeks, was stored on filter paper at -20 °C, 4 °C, room temperature, or 37 °C. Then FT₄ in these samples was measured as described in Methods. When the blood spots were left to dry at room temperature, the changes in FT₄ values did not exceed 15% on subsequent storage at either -20 °C, room temperature, or 37 °C (Figure 2). When the dried samples were stored at 4 °C for three weeks or more, the FT₄ approximately doubled. When the blood spots were not dried before storage, but instead immediately stored in closed packages, FT₄ values for all samples except those stored at -20 °C had increased by 30% or more by three weeks of storage.

As shown in Table 1, the within-assay coefficients of variation (CVs) were 6.0 to 9.0% (mean 7.6%) and the between-assay CVs were 5.6 to 7.1% (mean 6.4%) for dried blood samples stored at -20 °C.

**Comparison of enzyme immunoassay and RIA:** The FT₄ concentrations in 68 dried blood samples, as determined by the present assay (x) correlated well with those in serum samples as determined by RIA (y): \( y = 0.9x + 0.28; r = 0.98; p < 0.001 \).

FT₄ concentrations in various subjects: FT₄ concentrations in dried samples of normal blood as determined by the present method were 6.5 to 14.9 (mean 10.7) ng/L for adults, 9.8 to 20.7 (mean 15.2) ng/L for neonates. As shown in Figure 3, concentrations of FT₄ in dried blood samples from hyperthyroid subjects were high, ranging from 36 to more than 90 ng/L; those from subjects with primary and secondary hypothyroidism were low, less than 1.9 ng/L; and those from normal pregnant women (6.5 to 13 ng/L) or subjects with low TBG (adults: 6.5 to 13.5 ng/L; neonates: 11.2 to 16.5 ng/L) were within the corresponding normal ranges.

**Discussion**

Recently we developed a double-antibody enzyme immunoassay for determining FT₄ in serum (12). Here, we have used this method to determine FT₄ in dried blood samples on filter paper. The FT₄ in these samples was stable when stored at -20 °C, and we recommend storage at this temperature. When the samples were dried before storage, FT₄ was stable for at least four weeks at either room temperature or 37 °C. Therefore, properly prepared samples may be sent by mail. When the dried blood samples were stored at 4 °C, or the blood spots were not dried before storage and immediately stored unfrozen for more than two weeks, the

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*Equivalent to ng/L concentrations in serum; see text.*
FT₄ values increased significantly. Degeneration of TBG or alteration of T₄-TBG binding, or both, may be responsible for the changes in FT₄ concentration under these conditions of high humidity, but the mechanism is unclear. When the proposed method is applied to mass screening for neonatal hypothyroidism the cutoff point for FT₄ should be carefully determined in relation to stability of FT₄ during transportation and interassay variability of the method. The reproducibility of our method for determining FT₄ in dried blood samples on filter paper (within-assay CV 7.6%, between-assay CV 6.4%) was comparable with that of RIA: within-assay CVs 5.0-5.3%, between-assay CV 6.2% (8). The reliability of the proposed method was satisfactory, as shown by the good correlation of its results with the FT₄ values for serum samples determined by RIA.

The T₄-analog used in RIA (8) does not bind to TBG, but binds to albumin, so the FT₄ values measured by the RIA are influenced by the albumin concentration in serum (9). The low FT₄ values determined by RIA in pregnancy (8) may be due to changes in albumin concentration. Hence, in the present method we used a T₄-galactosidase conjugate, which does not bind to either TBG or albumin (11). Moreover, a nonisotopic assay is preferable in screening programs.

Our method has the following advantages that make it useful for screening: (a) it can detect hyper- and hypothyroidism even in subjects with alterations of their albumin or TBG concentrations; (b) it seems suited for use on dried blood samples on filter paper sent by mail; and (c) it can be done in a routine laboratory.

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References