Evaluation of Homogeneous Enzyme Immunoassay of Serum Thyroxine with the Gilford 3500 Analyzer

Kenneth L. Cruse

I evaluated homogeneous enzyme immunoassay (EMIT®) of serum thyroxine with the Gilford 3500 analyzer. The samples are pretreated before quantitation on the Gilford 3500; the procedure thereafter is entirely mechanized. Forty-four samples may be analyzed per run. Precision and accuracy are good, and results correlate well with those by radioimmunoassay.

Homogeneous enzyme immunoassay, first introduced by Rubenstein et al. (1), has become a very important analytical tool in the clinical laboratory. Recently, Ullman et al. (2) described a method for so measuring serum thyroxine. Conjugation of a thyroxine derivative to pig-heart mitochondrial malate dehydrogenase (EC 1.1.1.37) results in powerful inhibition of enzyme activity, with an increase in "apparent" $K_m$ of the substrate. Addition of anti-thyroxine antibodies reactivates the enzyme. Thyroxine in a patient's sample competes with enzyme-labeled thyroxine for antibody binding sites and thereby decreases antibody-induced activation of the enzyme. NAD$^+$ and malate are used in the system; the reaction is monitored by observing the absorbance change at 340 nm (3).

An evaluation of the thyroxine enzyme immunoassay (EMIT, a registered trademark of the Syva Co., Palo Alto, CA) is presented with use of the Gilford 3500. After pretreatment of the samples, the procedure is completely mechanized.

Materials and Methods

Apparatus

Gilford 3500 (Gilford Instrument Laboratories, Oberlin, OH 44074).
Auxiliary Pipettor/Diluter (Gilford).

Reagents

All reagents were obtained from the Syva Co. The manual thyroxine configuration was used for this study.

Antibody reagent A. Reagent A contains antibodies to a thyroxine derivative. When reconstituted, the reagent contains a standardized preparation of $\gamma$-globulin from immunized sheep, NAD$^+$, a stabilizer, and a preservative (pH 5.2). The reagent is stable for 16 weeks when stored at 2-8°C, after initial room temperature equilibration.

Reagent A diluent. The diluent contains 0.15 mol of glycine per liter, with stabilizer and preservative (pH 5.0). Reagent A diluent must be used cold and is stable for 16 weeks when stored at 2-8°C.

Working reagent A. Prepare by combining one volume of antibody reagent A with nine volumes of cold reagent A diluent. Allow working reagent A to equilibrate at room temperature for 8 h (or overnight) before use. Working reagent A can be used for one week when stored at 25°C. Each assay requires 0.5 mL of working reagent A.

Enzyme reagent B. Reagent B contains thyroxine chemically coupled to malate dehydrogenase in a 400/600 (by vol) mixture of glycerol and phosphate buffer (40 mmol/L, pH 9.2), with stabilizers and preservatives. Enzyme reagent B is stable for 16 weeks at 2-8°C.

Enzyme working reagent B. To ensure stability, enzyme working reagent B is prepared during the sample-pretreatment period of the assay. Prepare it by combining one volume of enzyme reagent B with one volume of de-ionized water. Each assay requires 110 μL of enzyme working reagent B. At the end of each run, any excess must be discarded.

Serum pretreatment reagent. A solution containing NaOH (0.5 mol/L) with "Liplex" (a proprietary sequestering agent for free fatty acids) is provided.

Pretreatment working reagent. Prepare by combining one part serum pretreatment reagent with four parts of de-ionized water. The solution is stable for one week at 25°C. Each assay requires 250 μL of pretreatment working reagent.

Thyroxine buffer. The buffer is a 0.3 mol/L solution of
glycine, containing 0.3 mol of L-malic acid (substrate) per liter, with preservatives (pH 9.9).

**Working thyroxine buffer.** Prepare by adding 25.0 mL of de-ionized water to the entire volume of thyroxine buffer supplied in the kit. Each assay requires 1.0 mL of the working thyroxine buffer.

** Thyroxine serum calibrators.** These are provided by Syva as separate lyophilized serum-based preparations. Reconstituted, they are stable for 16 weeks at 2–8 °C. Concentrations are: 20, 40, 80, 120, and 200 μg of L-thyroxine per liter.

**Procedure**

Allow all reagents to equilibrate to room temperatures. Set the Gilford 3500 as follows:

- **Temperature:** 25 °C
- **Sample time:** 2
- **Mode:** Absorbance
- **Vacuum:** 10 inches of Hg
- **Tower 2:** Position 9
- **Dispenser A:** Sample syringe 100 μL (no stop)
- **Dispenser B:** 0.5 mL (20% stop)
- **Wavelength:** 340 nm

Patients' samples and calibrators should be run in duplicate. Using a pipetter/diluter, place 50 μL of calibrator or sample, followed by 250 μL of working pretreatment solution, into a macro cup strip (blue). Position no. 1 of the first macro cup strip serves as a dispensing equilibration step; begin with position no. 2. Allow samples and calibrators to pretreat for 15 min at room temperature.

During the pretreatment period, prepare only enough of working reagent B for the run in progress (110 μL per assay). Prime the Gilford 3500 Dispenser A with working thyroxine buffer. Cycle at least five times. Prime Dispenser B with working reagent A. Recycle the reagent 10–15 times. Attach the tubing from Dispenser B to a stationary probe on Tower 2. The reason for longer recycling is to allow nonspecific binding of thyroxine antibodies to the inner surfaces of the tubing, a measure that results in better precision.

Enter the EMIT T4 program card into the card reader. When "READY" appears, press the "RUN" button. Follow the preamble. Stop when the preamble reaches SLEW.

Place pedestrians serum cups filled with 110 μL of working reagent B on the Gilford 3500 transport racks. At the end of the pretreatment period, place the macro cup strips (blue) containing pretreated samples and calibrators on the Gilford 3500 transport racks with the END-OF-RUN detector in place.

Press RUN; the Gilford 3500 will automatically move each pretreated sample into position and add working reagent A. During the second cycle, working reagent B and thyroxine buffer will be added to the final reaction mixture. The Gilford 3500 will then read, record, and print the initial and final absorbance and calculate each sample's EMIT unit.

**Results**

**Standard curve.** A standard curve may be constructed by averaging the EMIT units for each calibrator and plotting the results on the logit paper supplied by the Syva Co. The absorbance range between the 20 and 200 μg/L standards averaged 320 EMIT units, corresponding to 0.320 A.

**Precision.** Within-run precision was evaluated by analyzing three samples 30 times on the same day (Table 1). Between-run precision was studied by analyzing "Tri-rac I, II, and III" (Dade, Division of American Hospital Supply Corp., Miami, FL 33152) on each of 13 separate days (Table 1).

**Accuracy.** Accuracy of the assay was reflected in analytical recovery. The samples were prepared from patients' specimens pooled and supplemented (Table 2). Statistical analysis of recovery sample revealed: Gilford 3500 = 0.923 (theoretical) + 3.1 μg/L; r = 0.99.

**Clinical samples.** 151 patients' samples were assayed both by radioimmunoassay (Nuclear Medical Laboratories, Dallas, TX 75247) and the homogeneous enzyme immunoassay. The samples encompassed a range of concentrations from 10 to 200 μg/L. A linear regression analysis yielded the following: (y = homogeneous enzyme immunoassay; x = radioimmunoassay) y = 1.004x – 1.35 μg/L; r = 0.972, SEE = 0.59.

**Result analysis.** Of the patients' samples, 147 (97.3%) showed clinical and statistical agreement (both methods provided results within 2 SD of the correlation plot). The categories were: 25 patients (16.5%) hypothyroid, 101 (66.3%) euthyroid, and 25 (16.5%) hyperthyroid. The clinical status of all the subjects was verified.

Four samples (2.7%) showed discordance. Three samples lay slightly outside 2 SD from each other. Of these, one was in the normal range and two were in the high abnormal range. All three patients were verified as being euthyroid or hyperthyroid. No clinical confusion would have resulted from the variance. The remaining sample that was outside 2 SD was normal by radioimmunoassay but below-normal by enzyme immunoassay. Clinically, the patient was euthyroid.

The normal range for the radioimmunoassay of thyroxine was 45–125 μg/L.

**Discussion**

The convenience, precision, and accuracy of the homoge-

---

### Table 1. Precision Data

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>Normal</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, μg/L</td>
<td>28</td>
<td>59</td>
<td>157</td>
</tr>
<tr>
<td>Standard deviation, μg/L</td>
<td>1.6</td>
<td>3.1</td>
<td>6.4</td>
</tr>
<tr>
<td>CV, %</td>
<td>5.6</td>
<td>5.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Number</td>
<td>31</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Range, EMIT units</td>
<td>22</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>μg/L</td>
<td>24–31</td>
<td>53–65</td>
<td>140–165</td>
</tr>
</tbody>
</table>

### Table 2. Analytical Recovery Data (10 Samples) for Present Method and Radioimmunoassay

<table>
<thead>
<tr>
<th>Theoretical value</th>
<th>Gilford 3500</th>
<th>RIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroxine, μg/L</td>
<td>44</td>
<td>46</td>
</tr>
<tr>
<td>64</td>
<td>63</td>
<td>62</td>
</tr>
<tr>
<td>144</td>
<td>137</td>
<td>133</td>
</tr>
<tr>
<td>64</td>
<td>64</td>
<td>63</td>
</tr>
<tr>
<td>94</td>
<td>91</td>
<td>93</td>
</tr>
<tr>
<td>79</td>
<td>79</td>
<td>86</td>
</tr>
<tr>
<td>129</td>
<td>110</td>
<td>121</td>
</tr>
<tr>
<td>119</td>
<td>119</td>
<td>123</td>
</tr>
<tr>
<td>179</td>
<td>163</td>
<td>157</td>
</tr>
<tr>
<td>170</td>
<td>170</td>
<td>66</td>
</tr>
</tbody>
</table>
nenous enzyme immunoassay of thyroxine make it an attractive alternative to radioimmunoassay. The EMTT assay for thyroxine has been adapted to a large, multichannel discrete analyzer (4) and the ABA 100 Bichromatic Analyzer (5). The described procedure could readily be used by owners of the Gilford 3500 if their instrument contains a 1336 x 4 Configuration E Board or has a serial number greater than 700. Thyroxine analyses can be done at the rate of 56 patients' samples per hour, with precision and accuracy comparable to that for radioimmunoassay.

References
1. Rubenstein, K. E., Schneider, R. S., and Ullman, E. F., "Homoge-
3. Jakitsch, A. P., Schneider, R. S., Johannes, R. J., et al., Homoge-

CLIN. CHEM. 25/8, 1471–1475 (1979)

Four Electrophoretic Methods Compared for Diagnosis of Type III Hyperlipoproteinemia

Kanta Kuba, Kenneth Lippel, and Ivan D. Frantz, Jr.

Blood drawn from 192 probands and 1129 first-degree relatives who were participants in a collaborative family study of hyperlipoproteinemia at nine Lipid Research Clinics was used to prepare aliquots of whole plasma and top (d <1.006 g/ml) and bottom (d >1.006 g/ml) ultracentrifugal fractions. Each aliquot was analyzed at a central laboratory by electrophoresis on paper, agarose, and polyacrylamide gel, and by a combined electrophoretic precipitation technique. The electrophoretograms were evaluated for the presence or absence of a "floating-beta" lipoprotein band. All four methods agreed completely for 92.3% of the samples. An additional 2.0% of the samples were in agreement for three electrophoretic methods, but the paper electrophoretic results were not interpretable. Another 1.9% were considered to be "floating-beta" positive by paper electrophoresis but negative by the other three electrophoretic methods.

Additional Keyphrases: lipoprotein - broad-beta disease - floating-beta disease - intermethod comparison

Type III hyperlipoproteinemia (broad-beta disease; floating-beta disease) is a genetically determined lipoprotein disorder. It is characterized by a unique plasma lipoprotein pattern, a typical distribution and type of xanthomas, accelerated atherosclerosis, and a good response to therapy. The pathognomonic feature of type III hyperlipoproteinemia is the presence of a lipoprotein complex, not usually detected in normal plasma, associated with very-low-density lipoprotein (VLDL) when that fraction is isolated by a single centri-

References
1. Lipid Research Clinic, Dept. of Medicine, University of Minnesota, Minneapolis, MN 55455.
2. National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20206.
3. Received Mar. 23, 1979; accepted May 30, 1979.