We measured concentrations of free thyroxin (FT₄) in serum by using two new two-step FT₄ assays—a solid-phase two-step radioimmunoassay, Spectria®, and a time-resolved fluorimunoassay, Delfia®—and compared the results with those by a two-step FT₄ assay (RIA-gnost®), a one-step FT₄ analog assay (Amerlex-M™), and FT₄ measured after equilibrium dialysis. The new FT₄ assays classified 30 hypothyroid and 43 hyperthyroid patients (untreated) well. In 138 patients with nonthyroidal illness (NTI) and in late pregnancy (n = 36), fewer subnormal FT₄ values were reported by Spectria (P < 0.001), Delfia (P < 0.001), and RIA-gnost (P < 0.01) than by Amerlex-M. The results of the Spectria and Delfia methods correlated with the results of the dialysis method (r = 0.76) in NTI patients and pregnancy, and were in better agreement with the clinical state than was FT₄ by Amerlex-M. The FT₄ values by Amerlex-M, but not by other methods, correlated with albumin concentration. We conclude that these new two-step methods present good alternatives for FT₄ analysis.

Additional Keyphrases: thyroid status • nonthyroidal illness • pregnancy • albumin • intermethod comparison

Measurements of the free thyroxin (FT₄) concentration in serum are clinically important, particularly in borderline thyroid disease and in conditions involving abnormal protein concentrations or protein binding of thyroxin (1, 2). Of the several FT₄ radioimmunoassays currently available commercially, the one-step analog methods have gained relatively wide acceptance. However, they are documented to give spurious results, as compared with equilibrium dialysis, as patients with nonthyroidal illness (NTI) (3, 4). Our aim in this study was to evaluate two newly introduced two-step (so-called "back-titration") FT₄ assays in clinical practice, particularly in pregnant women and in patients with NTI.

Materials and Methods

Subjects

Each subject’s thyroid status was established on the basis of clinical evaluation and measurement of thyrotropin (thyroid-stimulating hormone, TSH) and, if necessary, the TSH response after administration of thyroliberin (thyrotropin-releasing hormone). Reference ranges of FT₄ assays were established from measurements from 65 euthyroid subjects (33 men), mean age 34 (SD 9) years (range, 19 to 48 years). The diagnoses of hypothyroidism (n = 30) and hyperthyroidism (n = 43) were also influenced by FT₄ values measured by Amerlex-M® assay, because this result was available when the subjects were evaluated. None of the hyperthyroid patients had triiodothyronine thyrotoxicosis.

Pregnant women (n = 36) in the third trimester attending an antenatal clinic and inpatients with NTI (n = 138) were studied. Five groups were formed from NTI patients: group 1, patients with acute myocardial infarction (n = 16) or cerebral infarction (n = 15); group 2, patients with severe infections treated in the intensive care unit (n = 16); group 3, patients with acute leukemia (n = 15) or colorectal cancer (n = 14); group 4, patients with severe hepatic cirrhosis (n = 15) or chronic hemodialyzed renal failure (n = 13); and group 5, patients with epilepsy who were receiving phenytoin, carbamazepine, or both (n = 34). Blood was drawn in the morning. Serum was separated and kept at -20 °C until assayed.

FT₄ Assays

The two new immunoassay kits for FT₄ in serum we evaluated were the Spectria® FT₄ two-stage radioimmunoassay (Farmos Diagnostica, Turku, Finland) and Delfia®
FT₄ time-resolved fluoroimmunoassay (Wallac, Turku, Finland).

In the Spectria, pipet 100 μL of a serum sample and 1000 μL of the assay buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, [10 mmol/L, pH 7.4] into test tubes coated with rabbit anti-T₄ antibodies. Incubate at 20 °C for 30 min on a shaker, decant, and wash. Add 1000 μL of ¹²⁵I-labeled T₄ and incubate at 20 °C for 1 h on a shaker. Decant the liquid carefully and measure the bound radioactivity remaining in the tubes.

For the Delfia assay, pipet 25 μL of serum samples and 200 μL of mouse monoclonal anti-T₄ antibody in buffer solution (50 mmol/L Tris·HCl buffer at pH 7.7, containing 9 g of NaCl, 500 mg of Na₂HPO₄, 5 g of bovine serum albumin, 500 mg of bovine globulin, 100 μg of Tween-40, and 20 μmol of diethylenetriaminepentaacetic acid per liter) into microtiter strip wells coated with rabbit anti-mouse IgG. Incubate at 20 °C for 1 h on a shaker and wash four times, then add 200 μL of Eu-labeled T₄ and incubate for 4 °C for 30 min. Wash the strips six times with buffer solution. Add 200 μL of the enhancement solution (0.1 mol/L acetate buffer adjusted to pH 3.2 with potassium hydrogen phthalate, 15 μmol of 2-naphtholylfluoracetone, 50 μmol of tri-N-octylphosphine oxide, and 1 g of Triton-X 100 per liter) to dissociate the europium ions from the labeled T₄. Measure the fluorescence in a time-resolved fluorometer.

For comparison we used a previously available solid-phase two-stage RIA, RIA-gnost® (Behringwerke AG, Marburg, F.R.G.). This method involved incubation with a polyclonal solid-phase antibody and, after decantation, a second incubation with ¹²⁵I-T₄. The one-step solid-phase FT₄ RIA routinely used in our laboratory was the Amerlex-M (Amersham International plc, Amersham, Bucks., U.K.).

Equilibrium dialysis method. We diluted serum samples fivefold in a buffer (pH 7.4) containing, per liter, 10 mmol of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma, St. Louis, MO), 1 mmol of sodium phosphate, 0.1 mol of NaCl, and 1 g of sodium azide. We dialyzed this through Visking dialysis membrane against the diluting buffer for 20 h at 37 °C in a shaking water bath. Thereafter, we determined T₄ in the dialysate with a solid-phase time-resolved fluoroimmunoassay involving a mouse monoclonal anti-T₄ antibody (Medix Biotech Inc., Foster City, CA) and a solid-phase-bound second polyclonal rabbit antibody against mouse immunoglobulins (Dakopatts A/S, Glostrup, Denmark). The 95% nonparametric reference interval determined earlier for the dialysis method was 9.5–22.4 pmol/L (mean 14.2, n = 122) (5).

Because not all patients could be studied with all methods, Table 1 gives the number of patients for different assays and the mean (SD) results.

Table 1. Serum FT₄ (pmol/L, mean ± SD) Measured by Five Methods in Pregnant Women and in Patients with Nonthyroidal Illness

<table>
<thead>
<tr>
<th>FT₄ assay</th>
<th>Pregnant women</th>
<th>Groups of NTI patients*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Amerlex-M</td>
<td>10.5 ± 1.7 (36)</td>
<td>12.5 ± 2.6 (31)</td>
</tr>
<tr>
<td>RIA-gnost</td>
<td>7.2 ± 1.3 (36)</td>
<td>12.7 ± 3.9 (31)</td>
</tr>
<tr>
<td>Spectria</td>
<td>7.5 ± 1.6 (36)</td>
<td>12.3 ± 2.4 (31)</td>
</tr>
<tr>
<td>Delfia</td>
<td>9.1 ± 1.0 (8)</td>
<td>15.0 ± 2.3 (29)</td>
</tr>
<tr>
<td>Dialysis</td>
<td>10.5 ± 2.3 (36)</td>
<td>16.1 ± 5.1 (31)</td>
</tr>
</tbody>
</table>

* Groups are as described in the text and in Figure 1. No. of patients is listed in parentheses.

Other Assays

TSH in serum was determined with the Spectria TSH immunoradiometric assay (Farnows Diagnostica). The determination limit of this assay is typically 0.03 milli-int. unit/L (reference interval 0.40–4.50 milli-int. units/L). Total T₄ in serum was determined with a radioimmunoassay from Farnows Diagnostica. Albumin in serum was determined with a BNA immunonephelometric analyzer (Behringwerke AG).

Statistical analysis. Reference ranges for FT₄ were calculated by determining nonparametric 2.5 and 97.5 percentile limits (6). Pearson correlation coefficients were used for analysis of correlation between FT₄ and albumin concentrations and between different FT₄ methods. Chi-square test was used to compare number of subnormal FT₄ values in different NTI groups.

Table 2. Between-Assay Precision of the Free Thyroxin Assays

<table>
<thead>
<tr>
<th>FT₄ assay</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amerlex-M</td>
<td>7.3</td>
<td>0.5</td>
<td>6.2</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>43.9</td>
<td>2.2</td>
<td>4.9</td>
<td>50</td>
</tr>
<tr>
<td>RIA-gnost</td>
<td>3.6</td>
<td>0.4</td>
<td>12.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>12.9</td>
<td>1.6</td>
<td>12.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>46.6</td>
<td>4.3</td>
<td>9.1</td>
<td>6</td>
</tr>
<tr>
<td>Spectria</td>
<td>3.3</td>
<td>0.4</td>
<td>10.6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>14.3</td>
<td>0.6</td>
<td>4.1</td>
<td>6</td>
</tr>
<tr>
<td>Delfia</td>
<td>4.8</td>
<td>0.8</td>
<td>11.9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>18.8</td>
<td>2.2</td>
<td>11.6</td>
<td>10</td>
</tr>
<tr>
<td>Dialysis method</td>
<td>9.4</td>
<td>2.1</td>
<td>22.4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>19.9</td>
<td>1.8</td>
<td>8.8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>37.8</td>
<td>2.0</td>
<td>5.4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>78.2</td>
<td>4.4</td>
<td>5.6</td>
<td>6</td>
</tr>
</tbody>
</table>
Delfia were reported as normal in four cases (of 24) and serum FT₄ measured by dialysis (n = 11) was reported as normal in two cases (Figure 2). Total T₄ was an insensitive detector for hyperthyroidism; only 67% of hyperthyroid patients had a high concentration of total T₄ (Figure 1). FT₄ values measured by Delfia and RIA-gnost were high in all cases, but Spectria and the dialysis method gave both high and normal values (Figure 2).

Thyroid-function studies in NTI patients and in pregnant women. The TSH value was normal in sera from 92% of the

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**Table 3. Serum FT₄ (pmol/L) in 65 Euthyroid Subjects**

<table>
<thead>
<tr>
<th>FT₄ assay</th>
<th>Mean (SD)</th>
<th>Range</th>
<th>95% confidence interval*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amerlex-M</td>
<td>15.7 (2.3)</td>
<td>11.5-21.1</td>
<td>11.7-21.0</td>
</tr>
<tr>
<td>RIA-gnost</td>
<td>13.6 (2.5)</td>
<td>8.7-21.9</td>
<td>9.5-20.3</td>
</tr>
<tr>
<td>Spectria</td>
<td>11.3 (2.1)</td>
<td>7.0-18.2</td>
<td>7.3-16.6</td>
</tr>
<tr>
<td>Delfia</td>
<td>11.8 (1.7)</td>
<td>8.0-16.7</td>
<td>8.4-15.3</td>
</tr>
</tbody>
</table>

*Nonparametric 2.5 and 97.5 percentile limits.

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**Table 4. Mean (and SD) TSH, Total Thyroxin, and Albumin in Pregnant Women and Patients with Nonthyroidal Illness**

<table>
<thead>
<tr>
<th>Reference interval</th>
<th>Pregnant women</th>
<th>Groups of NTI patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH, Int. units/L</td>
<td>0.40-4.5</td>
<td>2.4 (1.3)</td>
</tr>
<tr>
<td>FT₄, nmol/L</td>
<td>70-150</td>
<td>117.9 (22.8)</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>30-60</td>
<td>29.4 (3.2)</td>
</tr>
</tbody>
</table>

---

Fig. 1. Serum TSH, total T₄, and FT₄ (measured by Amerlex-M) concentrations in patients with nonthyroidal illness (groups 1–5), pregnant women (Grav), and hypo- and hyperthyroid patients.

Groups in Figs. 1–3: 1, acute myocardial or cerebral infarction; 2, severe infection; 3, acute leukemia or colorectal cancer; 4, hepatic cirrhosis or renal failure; and 5, epilepsy.

Fig. 2. Serum FT₄ concentrations as measured by Spectria, Delfia, RIA-gnost, and dialysis in patients with nonthyroidal illness (groups 1–5), pregnant women (Grav), and hypo- and hyperthyroid patients.
pregnant women and in 79% to 94% of the subjects in different NTI groups (Table 4). Total $T_4$ was low for patients who had received anticonvulsants (group 5) or had hepatic cirrhosis or renal failure (group 4). The concentration of albumin was lowest in patients with severe infection (group 2).

Pregnant women had lower mean $FT_4$ values than did euthyroid subjects by all methods (Figures 1 and 2). The two-step $FT_4$ methods, Spectria and Delfia, and the dialysis method gave significantly fewer subnormal values than did the analog-based method ($P < 0.001$) in NTI groups and pregnancy. In these groups, lower subnormal values were measured by RIA-gnost ($P < 0.01$, Table 5). The fewest subnormal $FT_4$ values were measured by Delfia, but the total number of measurements by this method was less (n = 96). $FT_4$ measured by Spectria was increased in one patient with acute leukemia. $FT_4$ values by Delfia were above the reference limits in 33-44% of groups 1-4.

**Albumin-dependence of $FT_4$ results.** $FT_4$ measured by Amerlex-M was subnormal in 93% of patients with severe infection (group 2). In this group albumin concentrations in serum were found to correlate significantly with $FT_4$ measured with Amerlex-M ($r$ = 0.54, $P < 0.005$, $n = 16$) as well as with total $T_4$ ($r$ = 0.59, $P < 0.05$). In pregnant women, decrease in serum albumin concentration was associated with a concomitant decrease of $FT_4$ concentration measured by Amerlex-M ($r$ = 0.51, $P < 0.01$). Total $T_4$ concentration also correlated with albumin concentration ($r$ = 0.41, $P < 0.05$). No such correlation was found between serum albumin and $FT_4$ measured with the two-step assays Spectria, Delfia, or RIA-gnost.

**Correlation of $FT_4$ results by different methods.** We found a very weak correlation between results for NTI groups and pregnant women obtained by the dialysis method and the analog-based Amerlex-M ($r$ = 0.34, Figure 3). Correlation analysis showed that the $FT_4$ results measured by Spectria and Delfia assays correlated somewhat better with results of the dialysis method ($r$ = 0.76, in both) than with results of $FT_4$ assay by RIA-gnost ($r$ = 0.64, Figure 3) in these groups. The $FT_4$ results by Spectria and Delfia were compared with the dialysis method also in hyper-, hyp-, and euthyroid subjects, whose $FT_4$ concentrations cover a wider range than in subjects with NTI (Figure 4).

**Discussion**

Serum total $T_4$ has limitations in screening for hyperthyroidism (7). In our study 33% of hyperthyroid patients had a normal $T_4$ value. The two-step $FT_4$ methods classified hypothyroid patients well. Serum free $T_4$ measured by dialysis was normal in 18% of clinically hypothyroid patient and in 3% of hyperthyroid patients. However, technical problems are encountered in the dialysis method, and precision is not as good as that with the direct $FT_4$ methods (8).

Most of the currently available direct $FT_4$ methods seek to underestimate the increase in the free fraction of $T_4$ in serum of NTI patients (9-11). However, it has also been reported that $FT_4$ results measured with the two-step RIA agree with those of the reference method and are superior to one-step RIAs (4). This study demonstrated substantial differences in the performance of five $FT_4$ assays in different patient categories. Serum $FT_4$ of NTI patients were within reference limits more often when measured by the two-step methods than when measured by the one-step analog-based method, Amerlex-M. Although the correlations between different $FT_4$ methods were poor in NTI groups, the two-step methods correlated better with the dialysis method than with Amerlex-M.

In the $FT_4$ assays the equilibrium between free and protein-bound $T_4$ is changed after antibodies are added to the reaction mixture. The amount of free $T_4$ in the sample unchanged as long as less than a small percent of total $T_4$ is bound to the antibody (12); however, this may not be the case in samples with altered binding protein concentrations or affinity. Erroneous results may occur when samples with altered binding proteins are compared with the calibrator with normal binding proteins. This may explain the poor correlation of $FT_4$ values in NTI patients when the dialysis method and $FT_4$ methods are compared. In the two-step methods, Delfia and Spectria, another source of interference binding of labeled $T_4$ to serum proteins, is avoided. Back titration of unoccupied antibody binding sites occurs after careful washing of serum components from solid-phase.

One-step $FT_4$ assays with a $T_4$-analog tracer are sensitive to changes in albumin concentrations because $T_4$ analog is bound to albumin (3, 13-15). This problem has not yet been solved despite attempts to improve the RIA system by adding blockers that chemically inhibit the analog–albumin binding (16, 17). Also, $FT_4$ with Amerlex-M was albumin-dependent in the present study. Moreover, it correlated very weakly with $FT_4$ results measured by dialysis method in NTI and late pregnancy groups.

Thyroxin-binding globulin and total $T_4$ are increased in pregnancy, and $FT_4$ concentrations reflect thyroid status more accurately than does total $T_4$ (18). In this study...
subnormal values were obtained with all FT₄ methods. Because, as has been recently shown, low-normal concentrations of FT₄ are physiological in late pregnancy (19), it would be reasonable to use different reference values in late pregnancy.

The precision of the new two-step methods was acceptable. Interassay CVs obtained with the new methods, Spectria and Delfia, were about the same as those with the routinely used analog-RIA technique Amerlex-M. In patients with NTI and in pregnancy, the two-step methods

Fig. 3. Correlation of FT₄ results in NTI patients and pregnant women as measured by Amerlex-M, RIA-gnost, Spectria, and Delfia, with those by the dialysis method

Regression lines (——) are calculated from 138 NTI patients and 36 pregnant (Gray) women. (. . .), line of identity
were in better agreement with the clinical state than was
the analog method. In conclusion, these methods present a
clear improvement in the assessment of thyroid function,
particularly in patients with NTI.

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A Simple Method for Estimating Association Constants between Monoclonal Antibodies and
Derivatized or Native Antigens

Willfried Schramm,¹ Tony Yang,¹ Mark E. Meyerhoff,² and Leonidas G. Baches³

We describe a simple method for estimating the association constant between an antibody and an antigen, based on a
theoretical treatment of experimental dose–response data. Our method was used to calculate the association constants of
an anti-progesterone monoclonal antibody for native pro-

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