Multicenter Evaluation of Enhanced Chemiluminescence Labeled-antibody Immunoassay (Amerlite-MAB™) for Free Thyroxine

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The technical and diagnostic performance of the Amerlite-MAB™ enhanced luminescence assay for free thyroxine (FT₄) was assessed in a multicenter evaluation trial. The euthyroid central 95% reference range for FT₄ (1393 subjects) was 11.5–27.7 pmol/L (by cumulative frequency plot with two-tailed 2.5% cutoffs). Results (y) agreed with those of similar radioactive method (Amerlex-MAB™ FT₄) (x): y = 1.06 x + 0.54, Sₓᵧ = 335.5, n = 235). Mean within-assay precision (CV) in six centers was 5.7% at 6 pmol/L and 2.6% at 51 pmol/L FT₄; between-assay precision (CV) was 7.2% and 3.6%, respectively. Diagnostic performance was assessed in all patient groups usually encountered, including those with nonthyroidal illness and extreme binding-protein anomalies. In elderly euthyroid subjects, the proportion of above-normal FT₄ values exceeded that in younger age-groups. These increases often accompanied normal estimates of thyrotropin and, in some cases, might have arisen by interference from medication that is taken more often by the elderly.

Indexing Terms: immunoenzymatic assay/precision/bindings proteins/thyroid hormones in nonthyroidal illness/pregnancy/age-related effects.

Recent experience (1–4) with new one-step labeled antibody immunoassays for free thyroxine (FT₄) in serum or plasma has demonstrated their diagnostic superiority over radioactive analog tracer methods, especially in sera with abnormal binding affinities or albumin concentrations (1, 2, 4) or with autoantibodies to thyroxine (T₄) (1, 4). Conjugating the tracer with large antibody molecules improves assay performance, partly by eliminating effects from tracer residually bound to serum T₄-binding proteins (3). With T₄-analog tracers this is harder to achieve, unless the analog is also a protein conjugate (Amerlite FT4*™ assay product literature: Amersham International, Amersham, UK, 1987; and ref. 5); even so, problems remain with thyroid hormone autoantibodies (1).

The omission of albumin from assay buffers departs from the conditions of the corresponding radioassay (2–4, 6). Therefore, unlike that assay, substances in serum able to displace T₄ from the T₄-binding proteins are not neutralized by additional binding sites and can therefore affect FT₄ estimates. However, the labeled-antibody assay, because it contains no extra albumin to bind T₄, responds to serum dilution as expected on theoretical grounds (3, 7–10).

We report a multicenter trial of the Amerlite-MAB™ FT₄ immunoassay, giving special attention to circumstances in which endogenous T₄ binding is thought to be disturbed (11–16).

Materials and Methods

Trial Design

Six centers (three from the UK, two from Germany, and one from Sweden) entered the trial on behalf of Kodak Clinical Diagnostics (Amersham, UK). Additional data were obtained at the company’s laboratories with sera and accompanying functional diagnosis provided by a UK center that took no direct part in the trial. Each center received three lots of reagents and used prescribed protocols for the Amerlite™ chemiluminescence system. Each performed at least eight 96-microwell assays (see Amerlite-MAB Free T₄* assay product literature, 1991, for assay details). Calibrators and controls were used in each assay. All centers performed both technical and clinical studies.

Technical Studies

In each assay, the first 12 wells contained duplicates of six calibrators (nominal values 0, 6, 13, 26, 60, and 100 pmol/L). Three control sera (Amerlite Free Thyroid Hormone controls, nominal FT₄ values 5.5, 17, and 50 pmol/L) were also measured in duplicate in each assay, as were five in-house control sera specially supplied for the trial (nominal FT₄ 4.5, 16.5, 40, 90, and (pregnancy serum control) 18 pmol/L). Unknown (patients') specimens were measured in duplicate in the remaining wells.

Within-assay precision, drift, and sensitivity were assessed by including both the lowest-concentration calibrator (0 pmol/L FT₄), the Amerlite Free Thyroid Hormone controls, and in-house controls throughout an assay plate in a predetermined pattern: 10 duplicates of the lowest calibrator and 4 duplicates each of the Free Thyroid Hormone and in-house controls.

Assay drift was estimated by regression of individual control values against well number over a standard assay (96 wells). The assay detection limit was calculated from 20 measurements by each participant of the zero FT₄ calibrator. The mean – 2 SD value of the zero calibrator light signal, interpolated to FT₄ concentration, represented the detection limit of the assay. The mean limit from all participants was calculated as the root mean square (RMS) value.

Within-assay precision was measured by taking the
mean CV obtained by each participant for each of the control sera from the assays specially dedicated to this study. Each participant calculated between-assay precision from the data for each control in eight assays. Precision overall was derived from the RMS value of the individual center’s estimates.

Patients’ Samples

$FT_4$ values defining the central 95% reference range were obtained from specimens ($n = 570$) from the routine thyroid clinics (i.e., from nonhospitalized, ambulatory subjects) in the participating centers described above, and also from the prediagnosed specimens ($n = 823$) from ambulatory subjects supplied by another center but measured in-house. No patients were receiving thyroid-related treatment. All subjects were considered euthyroid by the biochemical thyroid-function tests in routine use and by clinical presentation. No patient on oral $T_4$ therapy, pregnant, or obviously ill from serious systemic illness unrelated to thyroid disease (and thereby requiring hospitalization) was included in these groups.

Subjects judged to be hypothyroid or hyperthyroid had received no antithyroid or supplementation treatment. The hypothyroid subjects had above-normal thyrotropin concentrations and clinical symptoms indicative of this condition. In each center’s routine $FT_4$ assay, values ranged from the lower limit of sensitivity to the euthyroid–hypothyroid borderline. Hyperthyroid subjects had suppressed thyrotropin concentrations (as determined by high-sensitivity assays) and symptoms clinically suggesting thyroidal hyperfunction. Here, in-house $FT_4$ estimates in each center ranged from the euthyroid–hyperthyroid borderline to greater than the highest-concentration calibrator in the assays routinely in use; thyrotropin concentrations were <0.1 mIU/L.

Blood samples were taken and the sera separated and stored at $-20^\circ$C, according to each center’s specific protocol. Other specially selected stored samples were also used by each center.

Methods

$FT_4$. The protocol for the Amerlite-MAB $FT_4$ assay is described elsewhere (17). The centers also measured $FT_4$, $T_4$, and thyrotropin by using established methods. Because each center’s central 95% reference range for $FT_4$ was similar, even by different methods, we could amalgamate the results. In addition, for each patient group, the mean, range, and spread of $FT_4$ values by the Amerlite kit were compared between those determined by the centers taking part in the trial directly and those in the sera analyzed by the manufacturer. In all cases, the results were so similar that we could combine them.

Thyroxine-binding globulin (TBG). This was measured by the Amerlite TBG assay (central 95% reference range, 13.3–28.3 mg/L).

Transthyretin. This was measured by immunoprecipitation, with use of the Incstar SPQ kit (Atlantic Antibodies, Reading, UK) on a Cobas Bio analyzer (Roche Diagnostic Systems, Welwyn Garden City, Herts, UK). The central 95% reference range was 171–363 mg/L.

Albumin. Concentrations of human serum albumin were measured by the brom cresol purple method (18). The central 95% reference range was 30–55 g/L (mean 42 g/L).

Data analysis. Curve-fitting techniques for $FT_4$ assays were by the in-house methods previously established in each center. For Amerlite-MAB $FT_4$ assays, curve-fitting parameters intrinsic in the system were used to perform data analysis automatically (product literature, 1991). The method of interpolation is thus common to all centers. The interpolated data were analyzed with standard statistical packages: SAS on the Microvax computer (Microvax, Basingstoke, UK), spreadsheets on Hewlett-Packard computers (Hewlett-Packard, Sunnyvale CA), and additional statistical analysis on an Apple Macintosh Plus computer (Apple Computer, Cupertino, CA). Reference ranges were obtained from (a) each individual center, (b) the collective results of the trial, and (c) analysis of results obtained by the manufacturer, as described above. An overall euthyroid central 95% reference range was calculated from all results ($n = 1393$). Population distributions were normalized, if necessary, after skewness and kurtosis analysis. Appropriate data transformations were used to obtain the reference range. Ranges were expressed as mean ± 2 SD or, by cumulative frequency plots, as the values between the 2.5% and 97.5% population percentiles.

Hypothyroid and hyperthyroid populations were expressed as one-tailed 100% cumulative frequencies. Regression analysis was performed by the debiased regression method according to Deming (19).

Results

Analytical Assessment

Precision. The RMS within-assay CV for eight replicates of controls run in each of two assays at each of the six centers (i.e., 96 determinations per control) were: 6.3% (4.5 pmol/L nominal $FT_4$); 6.3% (5.5 pmol/L); 4.2% (16.5 pmol/L); 5.0% (17 pmol/L); 2.8% (18 pmol/L pregnancy control); 2.6% (40 pmol/L); 2.7% (50 pmol/L); and 2.6% (90 pmol/L). The corresponding between-assay RMS CVs for the same controls were: 9.5%, 7.4%, 4.2%, 5.4%, 4.8%, 3.3%, 3.6%, and 3.3%, respectively.

Drift. Assay drift, calculated from the percentage change between controls measured at the beginning and again at the end of a full plate assay, varied from −2.1% to −5.7% for all controls, based on 12 determinations from the six centers.

Detection limit. The overall RMS mean detection limit for $FT_4$ was 0.73 pmol/L (95% confidence range 0.34–1.22 pmol/L).

Clinical Assessment

Central 95% euthyroid reference range. For 570 euthyroid subjects measured by the six external centers in the trial, the central 95% euthyroid reference range for $FT_4$ was 10.6–27.0 pmol/L (mean 18.9 pmol/L, log$_{10}$ transformation). Calculated with untransformed data, the
corresponding mean and range were 17.4 and 9.0–25.9 pmol/L; with the cumulative frequency cutoffs at 2.5% and 97.5%, they were 16.7 (median) and 10.4–28.7 pmol/L.

For all 1393 samples analyzed both by the manufacturer and the participating laboratories, the central 95% reference range was (a) 11.1–25.8 pmol/L (mean 16.9 pmol/L) by log_{10} x transformation; (b) 9.5–25.0 pmol/L (mean 17.3 pmol/L) for untransformed data; and (c) 11.5–27.7 pmol/L (median 16.6 pmol/L) by cumulative frequency cutoff analysis. In individual centers, reference ranges varied from 9.9–30.0 to 10.3–24.8 pmol/L, an indication that different criteria for patient selection and clinical judgment can affect the results.

There was a small “tail” of results (43 to 1393, 3.1%) with FT_4 values >26 pmol/L (Fig. 1), both in the external trial (24 of 570, 4.2%) and in the manufacturer’s studies (19 of 823, 2.3%); the highest value was 39.5 pmol/L. These subjects were considered euthyroid because of their normal thyrotropin values and no clinical impressions of thyroid dysfunction.

**Correlation of FT_4 estimates with patient’s sex and age.** FT_4 estimates from the combined trial were subdivided two ways: (a) according to sex and (b) according to decades of age, regardless of sex, from 0 to 100 years. For all samples (393 males and 1000 females) or for both sexes, the data were best normalized by a log_{10} x transform (Fig. 2). The mean for males was 17.2 pmol/L (95% range 11.8–25.0); for females 16.8 pmol/L (10.8–26.0). These means were not significantly different (P > 0.05).

Ranges from either the six external participants or estimates obtained in-house by the manufacturer were identical.

In Table 1 and Fig. 3, the results were subdivided into intervals of decades of life. The ages were known for 292 males (range 4–90 years) and 709 females (range 2–97 years). Subjects older than 70 years had an increased FT_4 estimate more often than younger subjects and, by the 9th decade, 9% of values were above normal; thyrotropin values were normal. In the male group, a polynomial quadratic correlation with age best fit the data: y (FT_4) = 19.3 − 0.115 x (age) + 0.001 x^2 (r = 0.21, P < 0.002, S_{yx} = 8.15). For females, the corresponding correlation was linear: y = 0.057x + 14.1 (r = 0.30, P < 0.001, S_{yx} = 24.0). Both were highly significant correlations, the female correlation with age being greater. On combining all data (n = 1001), a polynomial quadratic correlation gave: y = 17.5 − 0.07x + 10^{-5} x^2 (r = 0.29, P < 0.001, S_{yx} = 19.4); the upper 95% regression line was y = 22.6 − 0.017x + 1.7 × 10^{-7} x^2, the lower 95% regression line was y = 10.9 − 0.1x + 6.3 × 10^{-7} x^2.

**Discrimination of hypothyroid and hyperthyroid sera.**

Fig. 2. Euthyroid ranges for male (A) and female (B) subjects. See text for data normalization, derivation of 95% reference ranges, and mean values for each group.

**Table 1. Mean and central 95% reference range for euthyroid subjects, by age.**

<table>
<thead>
<tr>
<th>Age, years</th>
<th>n</th>
<th>Mean*</th>
<th>95% range</th>
<th>No. (%) exceeding 95% range</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–20</td>
<td>29</td>
<td>16.1</td>
<td>11.8–25.2</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>21–30</td>
<td>129</td>
<td>16.2</td>
<td>12.1–22.7</td>
<td>0 (0)</td>
</tr>
<tr>
<td>31–40</td>
<td>140</td>
<td>16.2</td>
<td>11.5–23.0</td>
<td>1 (0.7)</td>
</tr>
<tr>
<td>41–50</td>
<td>156</td>
<td>16.4</td>
<td>11.0–24.3</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>51–60</td>
<td>133</td>
<td>16.5</td>
<td>11.2–24.3</td>
<td>2 (1.5)</td>
</tr>
<tr>
<td>61–70</td>
<td>184</td>
<td>17.1</td>
<td>11.5–25.4</td>
<td>7 (3.8)</td>
</tr>
<tr>
<td>71–80</td>
<td>128</td>
<td>18.2</td>
<td>11.8–28.1</td>
<td>9 (7.0)</td>
</tr>
<tr>
<td>81+</td>
<td>102</td>
<td>19.3</td>
<td>12.3–30.2</td>
<td>9 (8.8)</td>
</tr>
</tbody>
</table>

* Calculated from the transformed data. The transformation log_{10} x represents the best normalization of data in all subcategories.
The assay's ability to discriminate overt hypothyroid and hyperthyroid sera from the lower and upper limits of the central 95% reference range was investigated with samples from 220 hypothyroid, 224 hyperthyroid, and 1393 euthyroid subjects (total n = 1837).

Four of 224 (1.8%) of hyperthyroid values (overall mean 61.5 pmol/L) and 8 of 220 (3.6%) of hypothyroid values (overall mean 6.7 pmol/L) lay within the central 95% reference range, calculated from the 2.5% and 97.5% cumulative frequency cutoffs (11.5–27.7 pmol/L) (Fig. 1). Also, 33 euthyroid values (2.4%) lay within the hypothyroid range and 35 (2.5%) lay within the hyperthyroid range. Most of the hypothyroid FT4 values (97.5%) were <10.7 pmol/L, and 97.5% of hypothyroid values were >29.5 pmol/L. The sensitivity and specificity of the assay for discriminating the hypothyroid group, based on the cumulative frequency 2.5% and 97.5% cutoffs, were thus 96.4% and 97.8%; for the hyperthyroid group, they were 98.2% and 97.5%, respectively. Other range-setting methods (e.g., by logx transformation) differed little in discriminatory power. By all methods, only ~2.5% of patients with overt thyroid dysfunction were incorrectly assessed.

Receiver-operating characteristic plots were calculated (20) to compare how well the hypothyroid and hyperthyroid groups were discriminated from the euthyroid subjects in the Amerlite-MAB and other assays used (see Table 2) throughout the range of assay sensitivities and specificities. At the hyperthyroid border, the assays performed similarly (mean Amerlite-MAB specificity range, by receiver-operating characteristic plot, in the region of population overlap 0.97–1; sensitivity range 0.95–1; other assays: mean specificity range 0.92–1 and sensitivity range 0.91–1). At the hypothyroid borderline, however, the Amerlite-MAB assay's performance often appeared to be distinctly better, although the relatively small number of patients in each center did not demonstrate statistical significance (specificity ranges: mean Amerlite MAB 0.93–1, Diagnostic Products Coat-a-Count 0.89–1; Kodak Amerlex-MAB 0.80–1, Boehringer E600 0.53–1, TBK 0.71–1, Pharmacia Delfia 0.68–1, Kodak Amerlite 0.96–1; sensitivity ranges: Amerlite-MAB 0.81–1, Coat-a-Count 0.56–1, Amerlex-MAB 0.56–1, E600 0.42–1, TBK 0.77–1, Delfia 0.91–1, Amerlite 0.96–1.

**Correlation with the users FT4 assays.** The results in each center were correlated with those from the user's routine assay method (Table 2). In all cases, correlation coefficients were >0.88, although the slope of the regression line often exceeded 1 and intercepts often differed significantly from 0. Especially with analog tracer assays, the above-normal FT4 estimates occasionally observed in the Amerlite-MAB assay in elderly subjects were rarely seen. This influenced the value of the correlation coefficient (e.g., with the Coat-a-Count analog immunoassay, Table 2).

**FT4 dependency on the concentrations of binding proteins.** TBG, transthyretin, and albumin concentrations were measured in 230 euthyroid sera, and TBG and albumin concentrations in a further 59 and 33 euthyroid sera, respectively. TBG concentrations (mg/L, x) did not correlate significantly (P > 0.05) with FT4 (y) measured in the Amerlite-MAB assay. Linear regression gave the relation y = 18.3 – 0.03x (r = 0.08, SE of slope = 0.019, S_yx = −3.17). Serum TBG concentrations ranged from 2 to 80 mg/L (central 95% reference range 13.3–28.3 mg/L). For transthyretin, the linear regression between FT4 (y) and the protein (mg/L, x) for 250 subjects was y = 19.9 – 0.1x (r = 0.15, P = 0.02, SE of slope = 0.004, S_yx = −23.35), which is of low significance. Transthyretin

Table 2. Correlations of results from Amerlite-MAB and user's FT4 assay in the external trial.

<table>
<thead>
<tr>
<th>User's assay (x)</th>
<th>n</th>
<th>Intercept</th>
<th>Slope</th>
<th>SE of slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coat-a-Count<strong>a</strong></td>
<td>173</td>
<td>−1</td>
<td>1.22</td>
<td>0.05</td>
</tr>
<tr>
<td>Amerlex-MAB<strong>a,b</strong></td>
<td>263</td>
<td>0.54</td>
<td>1.06</td>
<td>0.02</td>
</tr>
<tr>
<td>TBK<strong>a,c</strong></td>
<td>202</td>
<td>7.25</td>
<td>1.38</td>
<td>0.06</td>
</tr>
<tr>
<td>E600<strong>a,c</strong></td>
<td>171</td>
<td>−0.48</td>
<td>1.34</td>
<td>0.03</td>
</tr>
<tr>
<td>Delfia<strong>a,d</strong></td>
<td>295</td>
<td>5.62</td>
<td>1.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Amerlite<strong>a,b</strong></td>
<td>234</td>
<td>−2.36</td>
<td>1.03</td>
<td>0.01</td>
</tr>
<tr>
<td>IMX<strong>a</strong></td>
<td>55</td>
<td>1.75</td>
<td>1.04</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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a Diagnostic Products Corp., Los Angeles, CA.
b Kodak Clinical Diagnostics Ltd., Amersham, UK.
c Boehringer Mannheim, Mannheim, Germany.
d Pharmacia Diagnostics AB, Uppsala, Sweden.

Abbott Diagnostics, N. Chicago, IL.
concentrations ranged from 118 to 450 mg/L (central 95% reference range 171–363 mg/L).

For FT4 vs albumin (x, g/L), the corresponding linear regression for 283 subjects was \( y = 22.3 - 0.1x \) (\( r = 0.12, P = 0.04, \text{SE of slope} = 0.048, S_{yx} = -2.35 \).). Again, the reverse correlation was of low significance: 0.05 > \( P > 0.01 \). Albumin concentrations ranged from 27 to 56 g/L (central 95% reference range = 30–55 g/L).

Finally, the total binding potential (TBP), as the sum of the product of concentration \( \times \) affinity constant for all three proteins, was calculated with use of accepted constants \((21, 22)\). This weight the potential contribution of each of the binding proteins of FT4 binding, as happens naturally. The TBP for each serum in which the concentration of all three proteins was measured \((n = 250)\) was then correlated with FT4. The linear regression was \( y = 22.2 - 0.04x \) (\( r = 0.18, P = 0.004, \text{SE of slope} = 0.014, S_{yx} = -9.03 \), where \( x = \text{TBP} \times 10^{-2} \). This inverse correlation, unlike those for individual binding proteins, is highly significant.

**FT4 in pregnancy.** FT4 estimates were obtained in all three trimesters of pregnancy from apparently healthy euthyroid subjects (Fig. 4). For 84 subjects in the first trimester, the data (Fig. 4A) were best normalized by a \( \log x \) transformation, giving a mean of 15.5 pmol/L FT4 (95% range 10.3–23.1). No values lay above the upper limit of the central 95% euthyroid reference range, and 3 of 84 (3.6%) lay below. The mean FT4 concentration was 92% of the euthyroid reference mean.

Corresponding results for 131 subjects in the second trimester (Fig. 4B) were (mean, \( \log x \) transform) 13.2 pmol/L (95% range 9.4–18.5 pmol/L), 78% of the euthyroid central 95% reference mean. No values lay above the euthyroid reference range, but 9 of 131 (6.9%) lay below.

For 128 subjects in the third trimester (Fig. 4C) the mean FT4 concentration (\( \log x \) transform) was 11.5 pmol/L (95% range 8.2–16.1 pmol/L), 68% of the euthyroid central 95% reference mean. For 38 of the 128 (29.7%), values were below the lower limit of the euthyroid reference range.

**FT4 estimates in nonthyroidal illness (NTI).** The category of severe NTI patients \((n = 303)\) comprised 288 subjects believed by clinical impression and overall thyroid function assessment (normal FT4 and thyrotropin) to be euthyroid; 6 believed, from high serum thyrotropin concentrations, low FT4, and clinical symptoms, to be hypothyroid; and 9 thought to be hyperthyroid, with below-normal thyrotropin by sensitive assays, high FT4, and clinical symptoms typical of the condition. The euthyroid group comprised subgroups of subjects with heart disease \((n = 38)\), liver disease \((n = 54)\), renal disease \((n = 111)\), uncontrolled diabetes \((n = 6)\), and other systemic disease states \((n = 79)\). Subjects believed to have thyroid dysfunction often had accompanying renal disease \((n = 8)\) or cardiac disease \((n = 7)\). Results are shown for all NTI subjects in Fig. 5.

The distribution of values was best normalized (as for all groups studied) by \( \log x \) transform (mean FT4 17.2 pmol/L, 95% range 9.6–30.8 pmol/L). Although this was close to the euthyroid reference mean, values were more...
widely spread than in the reference group: 10 values (3.5%) lay below the euthyroid reference range, 17 (5.9%) above. However, discrimination of hypothyroid and hyperthyroid subjects from the reference range was as effective as with non-NTI subjects.

The mean $FT_4$ estimates were: heart disease, 20.0 pmol/L; liver disease, 18.6 pmol/L; and renal disease, 16.1 pmol/L. These results are similar to those in the trial of the corresponding radioactive assay (Amerlex-MAB* $FT_4$) (23).

**Patients receiving oral $T_4$.** In 37 treated subjects considered to be clinically euthyroid the mean $FT_4$ concentration was 24 pmol/L (range 10.2–37.8 pmol/L), 142% of the untreated euthyroid reference mean. Broadening and extension of the range into higher $FT_4$ concentrations (Fig. 6), as is found by other assays (24), identified 8 subjects as being overtreated and 14 as undertreated (by thyrotropin measurements and clinical impression). The various categories overlap considerably, reducing the value of $FT_4$ measurements in this patient group (24).

**Drug and oral contraceptive therapy.** Apparently euthyroid subjects ($n=24$) taking phenytoin (Dilantin) gave a 95% reference range for $FT_4$ of 7.8–22.8 pmol/L (mean 15.3, SD 3.7 pmol/L). The mean was 89% of the untransformed central euthyroid reference mean. Two samples gave estimates below the euthyroid reference range. A slightly lower setting for $FT_4$ estimates is expected in this group (25).

For 29 pairs of patients' samples taken from subjects with chronic renal failure before and after heparin infusion in vivo, $FT_4$ measured after heparin infusion averaged 30% higher than before infusion. The range of preheparin values was 10.8–58 pmol/L vs postheparin, 9.9–82.5 pmol/L. For preheparin, only 2 of 29 (7%) of the results were above the central 95% euthyroid reference range; for postheparin, 11 of 29 (38%) were greater. More NEFA is probably produced in the latter in vitro after blood is withdrawn from the patient (15, 26–28), displacing more $T_4$ from the serum binding proteins, and thus increasing $FT_4$ (26–28).

For 19 apparently euthyroid subjects on aspirin (salicylate) antiinflammation therapy, the mean $FT_4$ was 17.8 pmol/L. This population was subdivided into two groups: 4 with $FT_4$ concentrations above the euthyroid reference range, and 15 with $FT_4$ estimates <18 pmol/L. The mean $FT_4$ of the larger subgroup was 14.8 pmol/L (95% range 10.1–19.4), only 88% of the euthyroid reference mean. We surmise that the smaller group had ingested salicylates shortly before blood sampling, leading to temporarily higher drug concentrations and thus increased displacement of $T_4$ from the serum binding proteins.

For 31 apparently euthyroid women taking oral contraceptives and thus having mildly increased serum concentrations of TBG, the 95% range and mean $FT_4$ were 8–23 and 15.3 pmol/L, similar to those of the reference euthyroid population.

**Familial dysalbuminemic hyperthyroxinemia (FDH).** Serum from 10 apparently euthyroid subjects with FDH had a mean $FT_4$ value of 12.4 pmol/L, 26% below the euthyroid reference mean. Three of the 10 values lay below the reference range; however, none was above the range, in contrast to the results with many analog tracer $FT_4$ assays. These results resemble those found by using equilibrium dialysis (29).

**Antithyroid hormone autoantibodies.** Seven samples contained high concentrations of anti-$T_4$ (or $T_3$) autoantibodies (these affect analog tracer assays severely). Two of these subjects were considered euthyroid (normal thyrotropin), two hypothyroid (increased thyrotropin), and three euthyroid taking oral $T_4$ therapy (normal or suppressed thyrotropin). $FT_4$ values were: 15.5 and 17.1 pmol/L, euthyroid; 9.7 and 11.9 pmol/L, hypothyroid; 27.6, 22.8, and 23.9 pmol/L, euthyroid on $T_4$ therapy. All values resembled those from similar subjects with no autoantibodies. In this, the assay agrees with the radiolabeled Amerlex-MAB* $FT_4$ method (1).

**Discussion**

The Amerlite-MAB $FT_4$ assay was evaluated to assess how it discriminated thyroid dysfunction and assigned subjects with highly abnormal concentrations or binding affinities of serum $T_4$-binding proteins to appropriate diagnostic regions. Because the assay buffers lack albumin, it was necessary to examine circumstances in which other substances might displace $T_4$ from the serum binding proteins. For example, subjects with severe NTI (11–16) could be taking drugs temporarily promot-
ing T₄ displacement (30–32) or inhibiting T₄-T₃ conversion (33).

In its normal reference range and discrimination of hypothyroid and hyperthyroid sera, the assay performed like its radioactive counterpart (3, 4). However, new relations emerged between FT₄ estimates and patients’ ages, not apparent formerly in FT₄ assays containing extra albumin (34). Before the 7th decade of life, FT₄ did not correlate with age in either sex (34). However, in elderly patients, ambulatory or hospitalized, a small but significant number of results lay well above the central 95% reference range. This was more often (but not always) found in female subjects. Because the thyrotropin concentrations accompanying the increased FT₄ findings were usually normal, these increases may be only transient. Indeed, in several cases, values quickly reverted to normal (R. Mardell, personal communication) without changes in thyrotropin content.

Although correlations of FT₄ with T₄-binding protein concentrations in euthyroid subjects were either statistically nonsignificant (TBG) or of low significance (trans-thyretin or albumin), FT₄ was more strongly correlated with the total binding capacity (TBP) of the serum proteins (concentration × T₄ binding constant). Interestingly, this forms a link with the well-known higher total T₄-binding capacity typical of hypothyroidism and the lower capacity typical of hyperthyroidism. Throughout the euthyroid reference range, the mean value of TBP fell by ~10%. Nonsignificant inverse correlations of transthyretin and albumin with FT₄ (partly due to small numbers of patients in the analyses) have been found by others (35). Similar trends emerged (unpublished results) in a two-step method for determining free thyroxine index.

In late pregnancy, FT₄ fell to ~70% of the nonpregnant euthyroid mean, with results close to the reference mean in the first trimester. This accords with other assays (analog or labeled antibody) with albumin in their buffers (6). This argues that lower serum FT₄ in pregnancy is real (6) and is not due to tracer binding by albumin (36). Nor could FT₄ be normalized by interferences in T₄ binding in vivo that extra albumin in assay buffers might reverse (36). Women in late pregnancy need specific FT₄ reference ranges to optimize discrimination of thyroid dysfunction (6, 37).

In NTI, the distribution of FT₄ values in subjects with no apparent thyroid dysfunction was broadly typical of the euthyroid reference range. Subjects may have (a) high FT₄/normal thyrotropin, (b) normal FT₄/high thyrotropin, or (c) normal FT₄/low thyrotropin. This suggests that both analytes must be measured to diagnose thyroid status in patients with severe NTI (23, 38, 39). The assay differentiated hypo- and hyperthyroid subjects from the euthyroid group, even with accompanying NTI.

In other respects, the assay performed as a valid FT₄ method should (7, 9, 40). Nonpregnant euthyroid subjects with abnormally high or low concentrations of TBG, transthyretin, or albumin in serum were measured accurately, as were subjects with the FDH syndrome or those whose sera contained antithyroid hormone autoantibodies. Additionally, the assay was precise in users’ hands throughout its working range, with minimal drift in results in a standard assay plate. Sensitivity was also acceptable, bearing in mind the clinically useful region of the assay.

In summary, the Amerlite MAB FT₄ assays shows fewer anomalies than earlier methods, even when substances (e.g., albumin) that might otherwise suppress the effects of interference are absent from assay buffers. In elderly subjects, there was evidence of fluctuating thyroid function. A hitherto unsuspected linkage emerged between FT₄ and total thyroid hormone binding capacity in euthyroidism. The assay performed well with patients with FSH syndrome, those with severe NTI, and those whose serum contained highly avid thyroid hormone autoantibodies. Together with good performance in all other patient categories and a greater robustness to serum dilution, assay speed and convenience were maintained. In all areas, results of the assay paralleled those of the accepted method of equilibrium dialysis and conform with theoretical expectations (7, 9).

References