Assessment of Serum Thyroxine Binding Capacity-dependent Biases in Free Thyroxine Assays

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Background: Free thyroxine (FT4) assays may exhibit biases that are related to serum T4 binding capacity (sBC). We describe two tests that can be used to assess the presence and magnitude of sBC-dependent biases in FT4 assays.

Methods: We used a direct equilibrium dialysis FT4 assay as the reference method and compared the results obtained with those of the FT4 assays under investigation, in patient sera having a wide range of sBC. We then compared the expected and observed FT4 results for sera diluted with an inert buffer. Because serum dilution causes a predictable decrease in sBC, an increasingly negative bias on progressive dilution is indicative of a sBC-dependent bias.

Results: The automated FT4 assay investigated (Vitros FT4) showed no demonstrable sBC-dependent bias by either test.

Conclusion: These two tests can be used to screen for sBC-dependent biases in FT4 assays.

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Irrespective of the thyroid function test strategy used, a measure of thyroid hormones is required to confirm the diagnosis of thyroid disease. The most appropriate measure of thyroid hormone is the moiety of thyroxine (T4),1 which is not bound by the thyroid hormone-binding proteins, and it is this free form of T4 (FT4) that is best correlated with thyroid status (1–3). Because the proportion of total T4 found in the free form is extremely small (~0.02% in euthyroid subjects), its quantification in the presence of the relatively very high concentrations of T4 bound to the binding proteins has proved exceedingly difficult. Indeed, an extensive literature exists highlighting interferences experienced with many commercially available FT4 assays (4–10). More recently, FT4 assays have become automated, which makes their use even more attractive to laboratories and has fueled the move from total to free thyroid hormone measurement. Although the newer FT4 assays are substantially better than the original assays, few seem to be as analytically accurate as the direct equilibrium dialysis (ED) FT4 method (9). Several studies have highlighted weaknesses in assay designs that produce significant biases (7, 9, 11, 12), the magnitude of which are assay specific and related to the concentration of the protein-bound T4 (PBT4). In particular, Nelson et al. (9) have shown that as the concentration of PBT4 decreased, the magnitude of the negative bias seen was increased. Careful examination of the experimental design adopted and data presented by Nelson et al. (9) suggested that the method-specific biases observed were dependent not only on the concentration of PBT4, but also on the serum binding capacity (sBC; calculated as the concentration × the affinity of free binding proteins).

In the present study, we examine the ability of two simple experimental tests in identifying the presence and magnitude of sBC-dependent biases in an automated FT4 assay (Vitros FT4). Both of these tests challenge the FT4 assays in the clinical situations outlined recently by the National Academy of Clinical Biochemistry (13), i.e., determination of the assay-specific biases in sera having a broad spectrum of binding protein abnormalities. For the first test, we used sera from patients having a wide sBC range. To ensure that the widest possible range of sBC was challenged, the test panel included sera from pregnant women in their third trimester, ambulatory subjects, and severely ill hospitalized patients because these groups previously have been shown to have high, normal,
and low sBC, respectively. For the second test, variation of sBC was achieved by the serial dilution of third trimester sera in an inert buffer.

**Materials and Methods**

The patient panel examined in the assays described below consisted of 26 ambulatory subjects, 18 women at the third trimester of pregnancy, and 25 severely ill patients who were admitted to an intensive care unit with a variety of illnesses, including sepsis, cardiac arrest, cardiac failure, and respiratory failure. All procedures used were in accordance with the Helsinki Declaration of 1975 (as revised in 1996). The patient sera were collected for routine analyses and were kept frozen (-20 °C) until required for the hormone measurements performed as part of the present study. All assays (with the exception of the ED FT₄, which was performed in the Cardiff laboratories of Ortho-Clinical Diagnostics) were carried out at the Clinical Biochemistry Laboratory of the Royal Infirmary, Edinburgh.

The sBC and PBT₄ concentrations of three sera were reduced in vitro by serially diluting (2- to 64-fold dilutions) sera in 10 mmol/L HEPES (Sigma Chemical; cat. no. H3375) buffer solution, pH 7.4. The sera chosen were from third-trimester pregnancies because these represented sera with high sBC and PBT₄ concentrations. The undiluted and diluted samples were assayed in the two FT₄ methods using standard protocols.

The automated FT₄ method used was the Vitros Immunodiagnostic Products (Ortho-Clinical Diagnostics, Amersham UK). The manual direct ED method was the Nichols FT₄ method (Nichols Institute Diagnostics). The physicochemical principles of the assays used are fundamentally different. The Vitros FT₄ assay is a labeled antibody method (2, 14), and the Nichols assay is a direct equilibrium method (15).

The ED method is carried out by dialyzing the FT₄ from 200 μL of serum into 2.4 mL of dialysis buffer (at 37 °C, over a 16- to 18-h incubation period). The FT₄ in the protein-free dialysate is then quantified by a sensitive solid-phase RIA for T₄. To minimize variability (e.g., assay-to-assay variation), all samples studied were analyzed on one occasion, with the samples randomized. The package insert quotes an intraassay CV at doses falling within and above the euthyroid range as being <13%. The euthyroid FT₄ range, which was observed with one outlier deleted from each end, quoted in the package insert is 10.3–34.7 pmol/L.

In the Vitros FT₄ assay, 25 μL of sample is pipetted into microtubes coated with a triiodothyronine (T₃)-protein conjugate, followed by 100 μL of a sheep anti-T₃ antibody labeled with horse-radish peroxidase in a 150 mmol/L phosphate buffer containing 1.0 g/L bovine gelatin and 1.0 g/L bovine γ-globulin. During the 16-min incubation at 37 °C, a proportion of the labeled antibody binds to the serum FT₄ and to the well surface, with the amount binding to the well surface being inversely related to the serum FT₄ concentration. The well is then washed, and a signal reagent that produces luminescence is then added; the resulting light emitted is measured in a luminometer. All procedures are carried out automatically by the Vitros ECI Immunodiagnostic system. The samples were assayed in a batch mode, with quality-control samples at three concentrations run at the beginning and end of the assay. The within-run imprecision for FT₄ values in the euthyroid and hyperthyroid range is quoted in the package insert as being <3%. The assay was calibrated using an in-house ED assay. The euthyroid range (1 and 99 percentiles) quoted in the package insert is 10–28.2 pmol/L.

In addition to the FT₄ assays, the patient sera were also analyzed in the Vitros Immunodiagnostic Products Total T₄ (TT₄) and T₃ uptake (T₃U) assays. The euthyroid T₄ range (2.5 and 97.5 percentiles) quoted in the package insert is 71.2–141 nmol/L. The T₃U assay is calibrated in %T₃U units, which are inversely related to the serum binding capacity (i.e., a serum with a high %T₃U has a low binding capacity). The euthyroid %T₃U range (2.5 and 97.5 percentiles) quoted in the package insert is 23.5–40.6% uptake.

All assays were performed following the manufacturers' instructions.

**Statistics**

Analysis of data was carried out by standard methods with Microsoft Excel spreadsheets. The dependence of the FT₄ bias observed with any variable was determined by correlating (linear regression analysis performed by the least-squares method) the methodological difference (from ED FT₄) of each patient (y-axis) against the variable studied (x-axis). The sBC was calculated by dividing the Vitros TT₄ concentration by the ED FT₄ concentration (9). The concentration of PBT₄ was estimated by subtracting the concentration of FT₄ from the concentration of FT₄, as measured by ED, from the concentration of TT₄ (9). Because the FT₄ concentration relative to TT4 was small (<0.1% of the TT₄), the PBT₄ concentration showed little difference from the TT₄ concentration. The agreement between the Vitros FT₄ and the manual ED FT₄ was tested by Deming regression (16). A measure of the sBC was also obtained by the T₃U, where the %T₃U is inversely related to the binding capacity. The Student unpaired t-test was used to compare the biochemical profiles between the patient groups (within each assay format), and the paired t-test was used when the comparison was across different assay methods.

**Results**

The quality-control sera used in each assay were all within expected ranges, and there was no evidence of drift in performance in any of the assays examined.

The relationship between the Vitros FT₄ and ED FT₄ assays in all patients studied is shown in Fig. 1. A significant correlation (r = 0.96; P <0.001) between the
Described by the following equations:

\[
\text{Vitros } FT_4 = 0.85 (\pm 0.03; P < 0.001) \quad \text{ED } FT_4 + 3.67 (\pm 0.61; P < 0.001).
\]

Good agreement between the FT4 methods was achieved in all patient groups.

The relationship between the Vitros FT4 to ED FT4 in (a) the ambulatory and pregnant subjects (groups combined, n = 44), and (b) the hospitalized patients (n = 25) is described by the following equations:

(a) \[
\text{Vitros } FT_4 = 0.9 (\pm 0.09) \quad \text{ED } FT_4 + 3.09 (\pm 1.19); \quad r = 0.83; P < 0.001
\]

(b) \[
\text{Vitros } FT_4 = 0.89 (\pm 0.07) \quad \text{ED } FT_4 + 2.33 (\pm 1.9); \quad r = 0.94; P < 0.001
\]

Table 1 shows the mean (± SD) and observed ranges for the two FT4 methods under investigation (FT4 in pmol/L), the TT4 (in nmol/L), %T3U, and sBC (in nmol/pmol) in the ambulatory, pregnant, and hospitalized patients. The FT4 concentration, as measured by both FT4 methods, in the hospitalized group was significantly (P < 0.001) higher than the FT4 concentration in the ambulatory group. None of the patients in the hospitalized group had FT4 concentrations below the corresponding euthyroid range. The calculated sBC in the hospitalized group was significantly lower (P < 0.001) than that found in the ambulatory group, whereas the corresponding %T3U value was significantly higher (P < 0.001). Thus, both measures of serum T4 binding support the view that hospitalized patients have decreased serum T4 binding capacities. The TT4 concentration in the hospitalized group was significantly lower (P < 0.001) than that obtained in the ambulatory group, and 12 of the 25 hospitalized patients had TT4 concentrations below the euthyroid range. The FT4 concentration in the pregnancy group was significantly lower (P < 0.001) than in the ambulatory group with both FT4 methods, whereas the sBC and TT4 concentrations were increased (P < 0.001). As expected, the %T3U was significantly (P < 0.001) reduced.

The FT4 concentrations obtained in the pregnancy group by the Vitros method were higher than the corresponding concentration obtained with the ED method. These differences, although small (mean difference, 2.6 pmol/L; range of difference in 95% confidence intervals, 2.1–3.2 pmol/L), were statistically significant (P < 0.001). Similar differences from ED FT4 were seen in the ambulatory group (mean difference, 1.6 pmol/L; range of differences in 95% confidence intervals, 0.7 to 2.6 pmol/L; both P < 0.001). The Vitros FT4 concentration in the hospitalized group was not significantly different (P > 0.05) from the mean concentration obtained in the ED method.

Fig. 2 depicts the relationship between the Vitros FT4 bias and sBC in all the patient samples. The regression equations derived in each individual category of subjects are shown below:

Ambulatory group: \[
\text{Vitros } FT_4 \text{ bias} = 0.77 (\pm 0.43) \quad \text{sBC} - 3.01 (\pm 2.62); \quad r = 0.34; P > 0.05
\]

Pregnant group: \[
\text{Vitros } FT_4 \text{ bias} = 0.167 (\pm 0.077) \quad \text{sBC} - 0.079 (\pm 0.167); \quad r = 0.475; P < 0.005
\]

Hospitalized group: \[
\text{Vitros } FT_4 \text{ bias} = -0.31 (\pm 0.665) \quad \text{sBC} + 0.29 (\pm 0.182); \quad r = 0.098; P > 0.05
\]

The slopes and intercepts of all the relationships did not differ significantly from each other (P > 0.05). The regression equation describing the relationship of the Vitros FT4 bias and sBC (the only individual patient category that

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**Table 1. Measured and calculated values in the three patient groups studied.**

<table>
<thead>
<tr>
<th>Category</th>
<th>Pregnant</th>
<th>Ambulatory</th>
<th>Hospitalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT4 (pmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>9.0 ± 1.7</td>
<td>14.3 ± 2.7</td>
<td>25.7 ± 9.1</td>
</tr>
<tr>
<td>Vitros FT4 (pmol/L)</td>
<td>5.8–11.6</td>
<td>9.7–23.7</td>
<td>14.2–48.6</td>
</tr>
<tr>
<td>Range</td>
<td>11.6 ± 1.7</td>
<td>15.9 ± 2.8</td>
<td>25.2 ± 8.1</td>
</tr>
<tr>
<td>TT4 (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>8.5–14.4</td>
<td>11.7–25.8</td>
<td>13.2–48.3</td>
</tr>
<tr>
<td>Vitros TT4 (nmol/L)</td>
<td>136.2 ± 23.1</td>
<td>84.7 ± 11.9</td>
<td>62.2 ± 24.1</td>
</tr>
<tr>
<td>Range</td>
<td>90.8–172.0</td>
<td>62.2–116.0</td>
<td>23.2–111.0</td>
</tr>
<tr>
<td>%T3U</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>18.9% ± 1.3</td>
<td>29.3% ± 2.18</td>
<td>47.5% ± 10.1</td>
</tr>
<tr>
<td>sBC (nmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>16.9–22.2</td>
<td>25.1–33.3</td>
<td>31.3–72.9</td>
</tr>
<tr>
<td>Calculated sBC (nmol/l)</td>
<td>15.5 ± 3.1</td>
<td>6.1 ± 1.1</td>
<td>2.6 ± 1.0</td>
</tr>
<tr>
<td>Range</td>
<td>10.7–23.9</td>
<td>4.2–8.5</td>
<td>1.2–4.5</td>
</tr>
</tbody>
</table>

* Significant (P < 0.001) difference from the corresponding ambulatory concentration.
yielded a significant slope and r value, both at P < 0.05, was the pregnancy group) indicates that at the highest observed sBC (23.9 nmol/pmol), the Vitros FT4 will be positively biased by 4 pmol/L (95% confidence interval, 2.6–5.4 pmol/L).

There was no apparent relationship (P > 0.05) between the PBT4 concentrations and the Vitros FT4 concentrations in any of the patient groups studied.

Fig. 3 depicts the relationship between the Vitros FT4 bias and %T3U in all patients studied. The equations derived in each of the individual patient categories are summarized below:

Ambulatory group: Vitros FT4 bias = -0.04 (± 0.22) %T3U + 2.75 (± 6.46); r = 0.035; P > 0.05
Pregnant group: Vitros FT4 bias = 0.003 (± 0.199) %T3U + 2.61 (± 3.78); r = 0.003; P > 0.05

Fig. 4. Mean percentage of change (from the undiluted sample) of ED (●) and Vitros FT4 (▲) after serial dilution of pregnancy sera with 10 mmol/L HEPES, pH 7.4.

Hospitalized group: Vitros FT4 bias = 0.06 (± 0.066) %T3U – 3.37 (± 3.18); r = 0.19; P > 0.05

None of these relationships reached statistical significance.

The results of the serum dilution experiment performed on the pregnancy sera are shown in Fig. 4. The theoretically derived FT4 concentrations suggest that the decrease after a 32-fold dilution will be <1%. Similarly, the FT4 concentration as measured by ED was only slightly affected by serum dilution. At dilutions of 1:16 and 1:32 (i.e., dilutions that are expected to decrease the sBC to levels usually seen in some hospitalized patients) the ED FT4 was within 5% of the concentration obtained in the undiluted sample. The Vitros FT4 concentrations at these dilutions did not vary significantly (<5%) from the FT4 concentration obtained in the undiluted sample.

Discussion

It is now accepted that FT4 shows a better correlation to the thyroid status than total T4 (1–3); thus it is the free hormone fraction that should be used to confirm the diagnosis of thyroid disease (17–19). The direct measurement of FT4 has, however, proved to be exceedingly difficult. Over the last two decades, numerous methodologies have been developed, and their limitations have been the subject of many publications and heated discussions in the literature (3, 7, 11, 20–28). Several limitations (e.g., “albumin” effects) of the early FT4 assays have been eliminated or minimized, but as shown recently, large methodological differences are still present (9). In their study, Nelson et al (9) measured FT4 (using different FT4 RIAs) in serum preparations in which the concentration of PBT4 (and sBC) was altered, whereas the ED FT4 concentration was kept constant. The results showed that the three FT4 methods studied were significantly, but to varying degrees, influenced by the serum PBT4. This method-specific dependency on PBT4 was proposed as a possible explanation for the discordant FT4 measurements seen in nonthyroidal illness. In an additional study, this
group of investigators (10) suggested that the method-
specific PBT 4 dependency was a direct result of increased
sequestration of T 4 by the assay reagents.

We have used two simple tests to examine the bias of
an automated (Vitros) FT 4 method. The first test involved
the comparison of the FT 4 results obtained in the Vitros
method in patients having a wide range of binding
capacities against those obtained in a commercial direct
ED system. The main reason for choosing ED as the
reference method is that it is one of the methods, in
addition to ultrafiltration, generally considered as the
“gold standard” method for free thyroid hormone mea-
surement. However, even these gold standards have their
own inconsistencies and technical weaknesses (15, 29–
32). Thus, the elevation of this particular ED method as
the definitive gold standard method is arguable. Nonethe-
less, the validity of the ED method chosen has been
well documented (15), although even this ED may be
biased in some nonthyroidal illness sera because of dilu-
tion effects during the dialysis step (33). The ED method
we used produces a 13-fold dilution of dialyzable sub-
stances in sera. The patient categories included in the
study have been documented to have high (i.e., third-
trimester pregnancy) and low (i.e., hospitalized patients)
binding capacities (13). This binding capacity profile has
been confirmed in the present study by two independent
methods (the sBC, derived by dividing the TT4 result by
the ED FT4 result, and the %T3U). The second test used
examined the effect of serum dilution on the FT 4 concen-
tration obtained by the methods under investigation
(Vitros and ED FT 4). The sample dilution test was chosen
for several reasons: (a) one can alter the sBC in a predict-
able fashion (e.g., a twofold dilution will reduce the sBC
by ~50%); (b) one can readily predict the ideal perfor-
amance of an assay and thus eliminate the need to compare
results with those obtained by a reference method; (c) one
can “manufacture” serum dilution pools whose sBCs
mimic the range of sBCs found in patients [e.g., the sBC
concentration range in patients undergoing thyroid test-
ing has been shown to be ~30-fold (13); thus dilution of a
pregnant serum by this factor will encompass the sBC
likely to be experienced by a laboratory]; and (d) this test
can easily be performed by any user.

The law of mass action (2, 3) dictates that the concen-
tration of FT 4 in serum depends on the equilibrium that
exists between the PBT 4 and the concentration and affinity
(i.e., the sBC) of the free binding sites:

\[ FT_4 = \frac{PBT_4}{sBC} \]

In estimating the sBC (i.e., PBT 4/FT 4) we made the
same assumption as Nelson and co-workers (9, 13), which
is that the FT 4 as measured by ED gives an unbiased
estimate of the true FT 4. As discussed previously, this
assumption is arguable. The highest and lowest individ-
ual sBC values in our study population were 23.9 and 1.2
nmol/pmol, which represent a range of 19.9-fold. Thus, it
is clear from this and other studies (9, 13) that patients
undergoing thyroid function tests possess a very wide
range of serum T 4 binding (and consequently wide ranges
of PBT 4). The results presented here show that the FT 4
concentrations obtained by the Vitros assay (relative to
ED), were not dependent on T 4 binding (sBC or T3U) or
PBT 4. The small positive bias (from ED) seen in pregnancy
is likely because of calibration differences. This is sup-
ported by the fact that the corresponding FT 4 concentra-
tions in the ambulatory group were also positively biased
to a similar degree.

In the second test, we examined the FT 4 biases of the
two methods (Vitros and ED FT 4) by analyzing sera
whose protein concentrations were decreased in vitro.
Lowering of the sBC by decreasing the concentration of the
binding proteins was accomplished by diluting sera in
an inert buffer (HEPES). The FT 4 concentrations as mea-
sured by both the ED and Vitros methods were not
reduced by the serum dilution.

The estimation of serum FT 4 by immunoassay, irre-

dispective of the methodology used will invariably disturb
the normal equilibrium between PBT 4 and sBC (2, 3). This
will come about as a result of the addition of the antibody
and the dilution of the sample in assay reagents that may
also contain T 4 binders such as albumin or animal sera,
which are often added to protect assays from interfer-
ces from heterophilic antibodies. These additions will
lead to the establishment of a new equilibrium and a new
“in vitro” FT 4 concentration. The concentration of this
in vitro FT 4 will be dictated by the in vitro bound T 4 and
unbound binding site concentrations. The in vitro bound
T 4 will be the net sum of PBT 4 and T 4 bound by the
immunoassay reagents (iPBT 4), and the in vitro unbound
binding sites will include, in addition to sBC, the free
antibody-binding sites and the free binding sites of other
binders included in the reagents [i.e., immunoassay bind-
capacity (iBC), which will be equal to the affinity ×
concentration of the free immunoassay binding sites].
Thus, the in vitro FT 4 will be equal to (PBT 4 + iPBT 4)/(sBC + iBC).
From this formula, one can predict that a
high iBC will cause biases that will be dependent on the
magnitude of the endogenous sBC. Thus, as the sBC
decreases (as seen in hospitalized patients and mimicked
by serum dilution), the assay will yield increasingly
negative results. The results presented show a lack of
sBC-dependent biases in both the Vitros and the ED FT 4
assays, suggesting that the in vitro disturbance of the
T 4/protein equilibrium induced by both assays is negli-
gible.

The tests described in the present study can be used to
assess the presence and magnitude of sBC-dependent
biases of other commercial FT 4 methods.

References

1. Robbins J, Rall JE. The interaction of thyroid hormones and protein