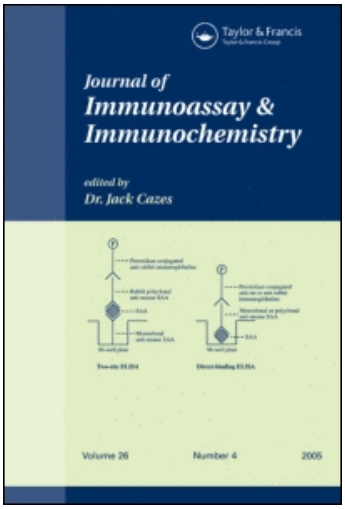

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Christofides, N. D. , Wilkinson, E. , Stoddart, M. , Ray, D. C. and Beckett, G. J.(1999) 'Serum Thyroxine Binding Capacity-Dependent Bias in an Automated Free Thyroxine Assay', Journal of Immunoassay and Immunochemistry, 20: 4, 201 — 221
To link to this Article: DOI: 10.1080/01971529909349351
URL: http://dx.doi.org/10.1080/01971529909349351

JOURNAL OF IMMUNOASSAY, 20(4), 201–221 (1999)

SERUM THYROXINE BINDING CAPACITY-DEPENDENT BIAS IN AN AUTOMATED FREE THYROXINE ASSAY

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ABSTRACT

The magnitude of serum thyroxine (T4) binding capacity (sBC) dependent bias in the AXSYM free thyroxine (FT4) assay was assessed using two recently described tests. One of the tests uses a direct equilibrium dialysis (ED) FT4 assay as the reference method. The results obtained with the AXSYM method were compared with those obtained by the ED FT4 method in patient sera having a wide range of sBC. The other test involves comparison of the FT4 results obtained following dilution of sera by an inert buffer, to theoretically derived FT4 results. As serum dilution causes a predictable decrease in sBC, the demonstration of a negative bias whose magnitude increases in parallel to the dilution, is indicative of an sBC-dependent bias. The AXSYM FT4 assay exhibited a significant sBC-dependent bias. This sBC-dependent bias is likely to have been caused by the presence of significant amounts of T4 binding proteins in the assay reagents.

(KEY WORDS: AXSYM Free Thyroxine, Binding capacity, Equilibrium dialysis, Protein-bound T4, immunoassay).

INTRODUCTION

The widely accepted free hormone hypothesis (1-3) states that it is the free thyroxine (FT4) rather than the protein bound T4 (PBT4) that can permeate cell membranes and elicit biological activity and that it is this free fraction that best correlates with thyroid function. Clearly, acceptance of the free hormone hypothesis, requires the use of accurate FT4 methods, i.e. assays which produce FT4 results which are independent of serum protein and PBT4 concentrations and thus do not exhibit FT4 biases in certain patient categories. However, because the proportion of FT4, relative to the protein bound T4, is extremely small (in euthyroid subjects it is approximately 0.02%) its quantification has proved exceedingly difficult. In the early radioimmunoassays (RIA) for FT4, the analog T4 tracers used were not sufficiently modified to prevent their binding to serum proteins, and as a consequence the FT4 concentrations obtained were significantly dependent on the concentration of albumin (and of other proteins) (4-8). Although some improvements in methodologies have been made, e.g. by better optimization of the chemistry of the analog tracers (9-10) or by changing the assay design to 2-step methods (2) and labelled antibody methods (2,11,12) it is clear that methodological differences still exist. In their study Nelson et al (13) showed that the FT4 concentrations obtained by three commercial RIAs were dependent (to different degrees) on the concentration of both the proteins and of the protein bound T4. It was also suggested (13-15) that method-specific biases (especially in sera with low protein bound T4 concentrations) were the result of increased sequestration of T4 by the assay reagents.

In the present study, we used two recently described tests (12) to assess the validity/accuracy of a fully automated FT4 method (the AXSYM FT4 assay). The tests used were specifically designed to challenge the presence of assay-specific FT4 biases in sera having different T4 binding capacities.

MATERIALS AND METHODS

Serum samples:

The patient panel, examined in the assays described below, consisted of 26 ambulatory subjects, 18 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 the procedures used were in accordance with the Helsinki Declaration of 1975 (as revised in 1996). The patient sera were collected for routine biochemistry tests and were kept frozen (-20^{0}C) until required for the hormone measurements performed as part of the present study.

The sBC and PBT4 concentrations of 3 sera were reduced *in vitro* by serially diluting (two-fold to 64-fold) sera in 10 mM Hepes (from Sigma; code H3375) buffer solution, pH 7.4. The sera chosen were from third trimester pregnancy as these represented sera with high sBC and PBT4 concentrations. The undiluted and diluted samples were assayed in the three FT4 methods using standard protocols.

The contribution of bovine serum albumin (BSA) in the generation of sBC-dependent FT4 biases was investigated by assaying two serum pools. One with normal sBC and another with low sBC, in FT4 assays whose reagents contained different BSA concentrations. The FT4 assay chosen for this investigation was the

Vitros ECi FT4 assay. Its antibody conjugate reagent, which does not contain BSA, was spiked with 3g/L, 10g/L and 30g/L of BSA (Biofac A/S, Copenhagen, Denmark).

Immunoassays

The Nichols (from the Nichols Institute Diagnostics, San Juan Capistrano, CA, USA) Equilibrium Dialysis (ED)method (16) was carried out by dialyzing the FT4 from 200ul of undiluted serum into 2.4ml of dialysis buffer (at 37^0 C, for 16 to 18 hr). The FT4 in the protein-free dialyzate was then quantified using a solid phase RIA for T4. In order to minimize variability (e.g. assay-to-assay variation and systematic bias), the samples were randomized and analyzed on one occasion. The package insert quotes an intra-assay CV at doses falling within, and above, the euthyroid range of <13%. The euthyroid FT4 range (which was the observed range with one outlier deleted from each end) quoted in the package insert is 10.3-34.7 pmol/l.

In the AXSYM FT4 assay (Abbott Laboratories, Diagnostics Division, Abbott Park, IL, USA), the sample and anti-T4 coated micro-particles are pipetted into a well. During this first incubation the antibody binds to serum T4. An aliquot of the reaction mixture is then transferred to the matrix cell, where the micro-particles bind irreversibly to a glass fiber matrix. The matrix cell is then washed to remove unbound material, and T3-alkaline phosphatase conjugate is added. During this second incubation, the conjugate binds to unoccupied (by T4) antibody binding sites. The matrix is then washed and the substrate is added. The resultant fluorescent product is measured by the Micro-particle Enzyme Immunoassay (MEIA) optical assembly. All the procedures are carried out automatically by the AXSYM system. The samples

were assayed in batch mode, with QC control material at three levels, run at the beginning and end of the assay to check for drift. The within-run precision for FT4 values in the euthyroid range is quoted in the package insert of < 6%. The euthyroid range quoted in the package insert is 9.1 to 23.8 pmol/l (the method used to calculate this range was not specified).

In the *Vitros* FT4 assay (12), 25 µl of sample is pipetted into micro wells which are coated with a T3-protein conjugate, followed by 100 µ l of a sheep anti-T4 antibody labeled with horse-radish peroxidase (HRP) in 150 mmol/l phosphate buffer containing 0.1% (w/v) bovine gelatin and 0.1% (w/v) bovine gamma globulin. During the 16 min incubation, at 37^0 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 is added which produces luminescence by the catalytic action of HRP. The resulting light emitted is measured in a luminometer. All the procedures are carried out automatically by the *Vitros ECi Immunodiagnostic* system. The within-run precision for FT4 values in the euthyroid and hyperthyroid range is quoted in the pack insert at < 3%. The assay was calibrated using an in-house equilibrium dialysis 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 ECi* Total T4 (TT4) and T3 uptake assays (T3U). In the TT4 assay 10 µl of serum is incubated with 80 µl of a sheep anti-T4 antibody and 80 µl of a T4-HRP conjugate

solution (the latter solution containing T4-displacing agents), in a micro well coated with a Donkey anti-sheep (DAS) secondary binder. The reactions occurring during incubation (15 min at 37^0 C) involve the uptake of the anti-T4 by the micro well surface and competition for antibody binding sites between the serum T4 and the T4-HRP. The euthyroid range (2.5 and 97.5 percentiles) quoted in the T4 package insert is 71.2-141 nmol/l.

In the T3U assay, 10 µl of sample is incubated (15 min at 37 C) with 80 µl of a solution containing a sheep anti-T3 antibody and 80 µl of a conjugate solution containing the T3-HRP conjugate tracer and exogenous T3. The exogenous T3 can be bound by both the serum binding proteins and by the antibody, whereas the T3-HRP can only be bound by the antibody. Thus, in a serum with low available binding capacity, a higher proportion of exogenous T3 competes with the T3-HRP for antibody binding sites which will result in a reduced binding of the T3-HRP. 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 range (2.5 and 97.5 percentiles) quoted in the package insert is 23.5-40.6% uptake.

All the assays were performed following the manufacturers' instructions.

Statistics:

Analysis of data was carried out by standard methods with Microsoft Excel spreadsheet. The agreement between the AXSYM FT4 method and the ED FT4 was tested by Deming Regression (17). The serum binding capacity (sBC) was calculated

by dividing the *Vitros* TT4 concentration by the ED FT4 (12,15). Another measure of the serum binding capacity was obtained by the T3U, where the % T3U is inversely related to the binding capacity. The sBCs of the samples used in the study of the effect of exogenous BSA, were calculated by dividing the TT4 concentration with the FT4 concentration obtained with the 0% BSA formulation. The concentration of PBT4 was estimated by subtracting the concentration of FT4, as measured by ED, from the concentration of TT4 (13,15). 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 dependence of the AXSYM FT4 bias observed with any variable was determined by correlating (linear regression analysis performed by the least-squares method) the difference between the FT4 concentrations obtained by the AXSYM assay and the values obtained by the ED FT4 assay in each patient (Y-axis) against the variable studied (X-axis). Students' unpaired t-test was used to compare the biochemical profiles between the patient groups (within each assay format) and a paired t-test 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.

Figure 1 depicts the relationship between the AXSYM FT4 and ED FT4 assays in all the patients (n=69) studied. A significant agreement, albeit with a rather poor correlation coefficient ($r = 0.69$, $p<0.001$) between the two assays was observed. The

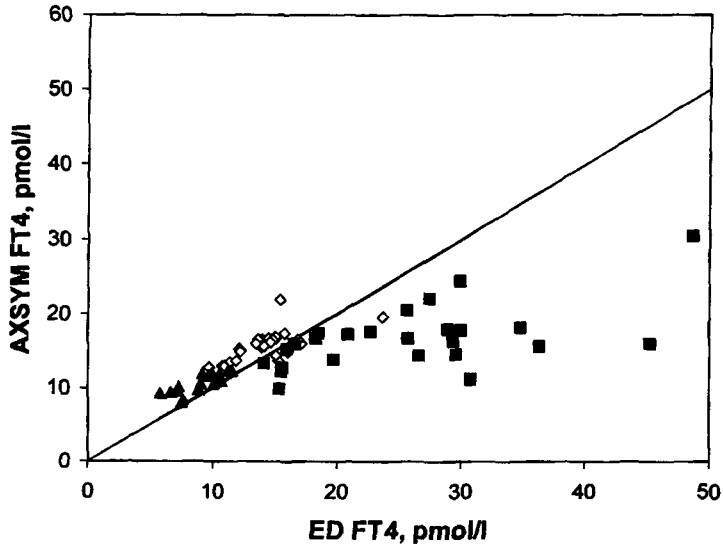

FIGURE 1. Relationship of the AXSYM and ED FT4 in ambulatory (depicted as diamonds), hospitalized (depicted as squares) and pregnant subjects (depicted as triangles).

equation describing the relationship was:

AXSYM FT4 = slope, 0.43 (+/- SEM, 0.055 $p<0.001$) ED FT4 + intercept, 7.45 (+/- SEM, 1.06, $p<0.001$).

The poor overall correlation appeared to be due to the fact that the FT4 in the hospitalized group showed particularly poor agreement between methods. The relationship of the AXSYM FT4 to ED FT4 in (a) the ambulatory and pregnant subjects and, (b) the hospitalized patients is described by the following equation:

a) AXSYM FT4 = 0.89 (+/- 0.07) ED FT4 + 2.87 (+/- 0.92), $r=0.88$ ($p <0.001$)

b) AXSYM FT4 = 0.47 (+/- 0.14) ED FT4 + 4.78 (+/- 3.82), $r=0.57$ ($p < 0.01$).

TABLE 1

Measured and Calculated Parameters in the 3 Patient Groups Studied.

	Pregnant	Ambulatory	Hospitalized
ED FT4 (pmol/l)	9.0 +/- 1.7*	14.3 +/- 2.7	25.7 +/- 9.1*
range	(5.8 to 11.6)	(9.7 to 23.7)	(14.2 to 48.6)
AXSYM FT4	10.6 +/- 1.4*	15.8 +/- 2.0	16.8 +/- 4.3
(pmol/l)	(7.9 to 12.5)	(12.8 to 21.9)	(10 to 30.4)
range			
VITROS TT4	136.2 +/- 23.1*	84.7 +/- 11.9	62.2 +/- 24.1*
(nmol/l)	(90.8 to 172.0)	(62.2 to 116.0)	(23.2 to 111.0)
range			
VITROS T3U (%	18.9 +/- 1.3*	29.3 +/- 2.18	47.5 +/- 10.1*
uptake)	(16.9 to 22.2)	(25.1 to 33.3)	(31.3 to 72.9)
range			
Calculated sBC	15.5 +/- 3.1*	6.1 +/- 1.1	2.6 +/- 1.0*
(nmol/pmol)			
range	(10.7 to 23.9)	(4.2 to 8.5)	(1.2 to 4.5)

* denotes a significant ($p<0.001$) difference from the corresponding ambulatory concentration

ED, equilibrium dialysis; sBC, serum binding capacity.

Table 1 shows the mean (+/- SD) and observed ranges by the AXSYM and ED FT4 methods (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 the ED method, in the hospitalized group was significantly ($p<0.001$) higher than the FT4 concentration in the ambulatory group. This contrasted with the profile obtained by the AXSYM FT4 assay, as the concentration in the hospitalized group was found to be not significantly different from the concentration obtained in the ambulatory group. The calculated mean 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 mean 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 mean FT4 concentration in the pregnancy group was significantly lower ($p<0.001$) than in the ambulatory group with both FT4 methods, whilst the sBC and TT4 concentrations were elevated ($p<0.001$). As expected, the % T3U was significantly ($p<0.001$) reduced.

The FT4 concentrations obtained in the pregnancy group in the AXSYM method, were higher than the corresponding concentration obtained in the ED method. These differences although, small were statistically significant (the mean differences and their 95% confidence intervals were, 1.6 pmol/l , range 1.1 to 2.1 pmol/l, $p<0.001$). Similar differences from ED FT4 were seen in the ambulatory group (the mean differences and their 95% confidence intervals were, 1.5 pmol/l, range 0.7 to 2.3 pmol/l, $p<0.001$). The mean AXSYM FT4 concentration in the hospitalized group was 8.9 pmol/l lower (95% confidence interval of 5.8 to 12 pmol/l, $p<0.001$) than the corresponding concentration obtained in the ED method.

Figure 2 depicts the sBC-dependent bias in the AXSYM FT4 values in all the patients. Although the regression statistics (e.g. $r = 0.54$, $p < 0.001$) suggest a significant sBC-dependent bias in the AXSYM FT4 assay, it is clear from the figure that this dependency is not linear. This non-linearity appears to be due to the large

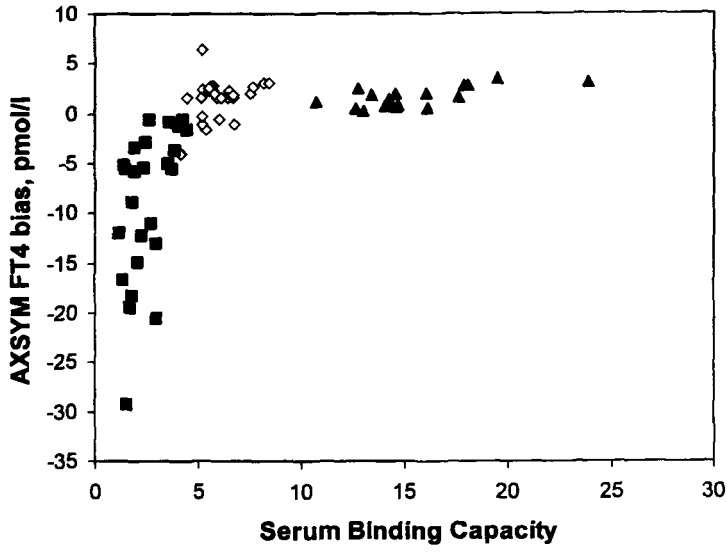

FIGURE 2. Relationship between the AXSYM FT4 bias (from ED FT4) and sBC in all patients (identified with the same symbols as in figure 1).

negative bias found in the hospitalized group. The data suggest that the bias exhibited by the AXSYM assay occurs when the sBC is decreased to <5 nmol/l. The regression statistics obtained in the different patient categories were:

Ambulatory subjects: AXSYM FT4 bias = 0.64 (+/- 0.365) sBC - 2.4 (+/- 2.243), r = 0.337 ($p > 0.05$)

Pregnant subjects : AXSYM FT4 bias = 0.21 (+/- 0.06) sBC - 1.68 (+/- 0.99), r = 0.64 ($p < 0.01$)

Hospitalized subjects : AXSYM FT4 bias = 3.96 (+/- 1.32) sBC - 19.1 (+/- 3.62), r = 0.57 ($p < 0.01$)

The slopes and intercepts obtained in the ambulatory and pregnant groups were similar ($p > 0.05$), but were significantly different from those in the hospitalized group (ambulatory versus hospitalized, $p < 0.02$ for slope and $p < 0.001$ for intercept, pregnant versus hospitalized, $p < 0.01$ for slope and $p < 0.001$ for intercept). The AXSYM FT4 bias was significantly ($p<0.01$) correlated with sBC in the pregnant and hospitalized groups but not in the ambulatory group. Using the equations describing the relationship between sBC and the AXSYM FT4 bias one can readily predict that at the highest individual sBC observed in the pregnancy group, (i.e. 23.9 nmol/pmol), the AXSYM FT4 assay will be positively biased by 3.4 pmol/l (95% confidence interval of 2.3 to 4.5 pmol/l). The calculated AXSYM FT4 bias at the lowest observed individual sBC in the hospitalized group (i.e. 1.3 nmol/pmol) would be -14.4 pmol/l, (95% confidence interval of 10.6 to 18.3 pmol/l).

The PBT4 concentration was significantly correlated to sBC ($r = 0.86$), and thus it was not surprising that the bias exhibited by the AXSYM FT4 method was also found to be significantly ($p < 0.01$) PBT4-dependent (AXSYM FT4 bias = 0.072 (+/-0.022) PBT4 -8.76 (+/-2.1), r=0.37).

Figure 3 depicts the relationship between the AXSYM FT4 bias and % T3U in all patient samples. The equation of this relationship and the regression equations derived in each individual patient category are shown below:

All patients : AXSYM FT4 bias = - 0.39 (+/- 0.043) % T3U + 10.7 (+/- 1.52), $r = 0.75$ ($p < 0.001$)

Ambulatory : AXSYM FT4 bias = 0.17 (+/- 0.18) % T3U - 3.58 (+/- 5.41), $r = 0.19$ ($p > 0.05$)

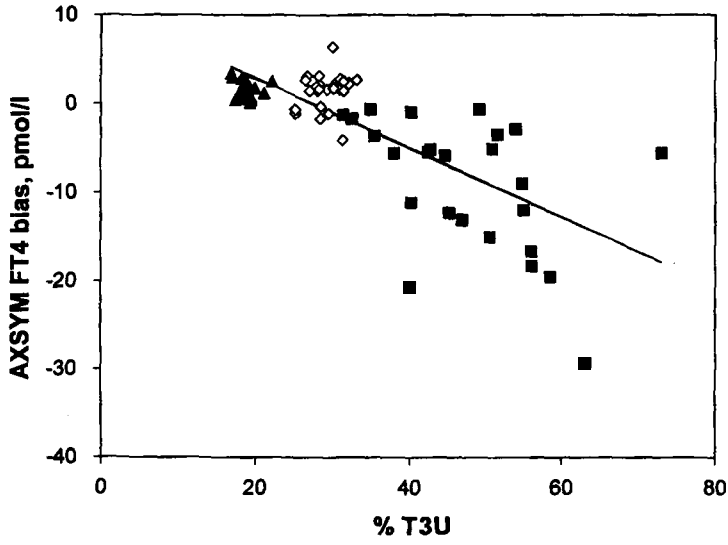

FIGURE 3. Relationship between the AXSYM FT4 bias (from ED FT4) and % T3U in all patients.

Pregnant : AXSYM FT4 bias = -0.12 (+/- 0.18) % T3U - 3.87 (+/- 3.9), r = 0.16 ($p > 0.05$)

Hospitalized : AXSYM FT4 bias = - 0.35 (+/- 0.137) % T3U + 7.9 (+/- 6.6), r = 0.47 ($p < 0.05$)

The highly significant ($p < 0.001$) correlation between the AXSYM FT4 bias and % T3U in all patients reflects the fact that the FT4 values for the hospitalized group (i.e. patients with high % T3U) are grossly biased (negatively). The values for the pregnancy and ambulatory groups (i.e. the sera having normal and low % T3U) are only marginally biased (positively). No relationship (slope, intercept and r, all $p>0.05$) between the AXSYM FT4 bias and % T3U was obtained in the ambulatory

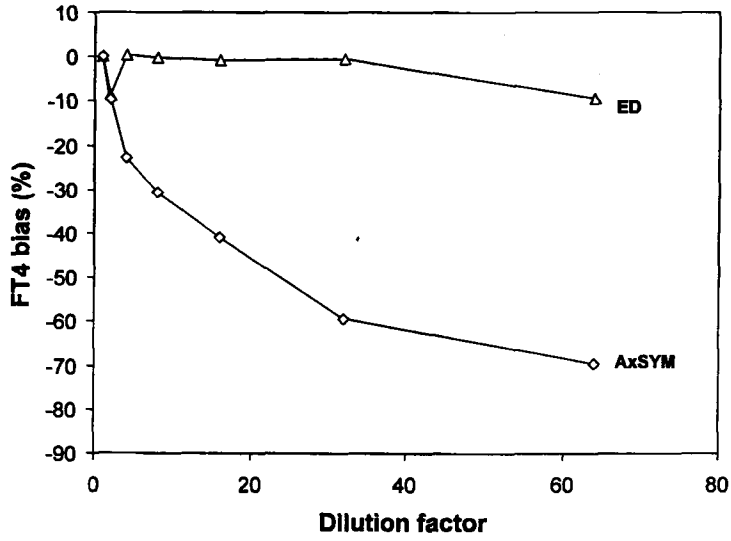

FIGURE 4. Mean percentage change (from the undiluted sample) of ED (triangles) and AXSYM (squares) following serial dilution of pregnancy sera with 10mM Hepes pH 7.4.

and pregnant groups. A significant correlation (slope, $p< 0.02$, r, $p<0.05$) was however, obtained in the hospitalized group. The equation obtained in the hospitalized group would predict that a serum with the highest % T3U observed (which was 72.9%) would be negatively biased by 17.6 pmol/l (95% confidence interval 10.7 to 25.1 pmol/l)

Figure 4 depicts the results of the serum dilution experiment performed on the pregnancy sera (i.e. sera with high sBCs). The theoretically derived FT4 concentrations suggest that the decrease following a 32-fold dilution will be <1%. Similarly, the FT4 concentration as measured by ED is little affected by serum

TABLE 2

Effect of Varying Amounts of Exogenously Added BSA on the FT4 Concentration of two Serum Samples Having Different Sbc Concentrations.

FT4 assay formulation	normal sBC (5.9 nmol/pmol) serum	low sBC (2.4 nmol/pmol) serum
0 g/l BSA	15.4 pmol FT4/l	35.2 pmol FT4/l
3 g/l BSA	17.1 pmol FT4/l	27.7 pmol FT4/l
10 g/l BSA	17.6 pmol FT4/l	25.2 pmol FT4/l
30 g/l BSA	20.0 pmol FT4/l	24.8 pmol FT4/l
AXSYM FT4 method	14.5 pmol FT4/l	16.3 pmol FT4/l

BSA, bovine serum albumin; sBC, serum binding capacity.

dilution. At dilutions of 1 in 16 and 1 in 32 (i.e. dilutions which are expected to decrease the sBC concentration to levels normally seen in some hospitalized patients), the ED FT4 was within 5% of the concentration obtained in the neat sample. Robustness to dilution could not, however, be demonstrated in the AXSYM FT4 assay, where the 16-fold and 32-fold dilution resulted in 41% and 59.4% decrease in the FT4 concentrations, respectively.

Table 2 shows the FT4 concentrations, of two sera having different sBCs, obtained in the different formulations of the Vitros FT4 assay. The results of the AXSYM FT4 assay are also shown. The FT4 concentration of the normal sBC serum became progressively elevated in parallel to the increase in the BSA concentration. The 30g/l BSA formulation yielded a FT4 result that was positively biased by 4.6 pmol/l (or +29.9%) from the FT4 result obtained using the formulation containing no BSA. In contrast, increasing the concentration of BSA resulted in a negative bias in the serum with low sBC. The magnitude of the negative bias was dependent on the

concentration of the added BSA. The 30g/l BSA formulation yielded a FT4 result that was negatively biased by 10.4 pmol/l (or -29.5%) from the FT4 concentration obtained using the formulation containing no BSA. The % difference between the FT4 concentration in the low sBC serum from that obtained in the normal sBC serum, in the formulation containing no BSA was 129%, whilst the % difference in the formulation containing 30g/l BSA was just 24%. The corresponding value for the AXSYM FT4 method was 12%.

DISCUSSION

It is now generally accepted that FT4 shows a better correlation to the thyroid status than total T4 (1-3) and thus it is the free hormone fraction, which should be used to confirm the diagnosis of thyroid disease (18-20). The major limitation of total T4 measurement arises because its concentration is strongly influenced by the concentration of the thyroid transport proteins (thyroxine binding globulin, albumin and transthyretin). For example, following administration of estrogen containing oral contraceptives both the thyroxine binding globulin and TT4 concentrations will increase, whereas a reduction in TT4 will occur in any situation where the protein concentration is reduced. Thus, a major requirement for a FT4 assay is the lack of any protein concentration (or capacity) dependency (1-3).

We have used two simple tests to examine the biases of the AXSYM FT4 method. The first test involved the comparison of the AXSYM FT4 results obtained in patients having a wide range of binding capacities against those obtained in a

commercial direct equilibrium dialysis FT4 assay. The main reason for choosing ED as the reference method is that it is one of the two methods (in addition to ultra filtration) generally considered as the "gold standard" method for free thyroid hormone measurement. The patient categories included in the study have been documented (15) to have high binding capacities (i.e. third trimester pregnancy), and low binding capacities (i.e. hospitalized patients) (13). This binding capacity profile has been confirmed in the present study by two independent methods (sBC, derived by dividing the TT4 by the ED FT4, and % T3U). The second test used, examined the effect of serum dilution on the FT4 concentration obtained by the methods under investigation. As discussed previously (12) the sample dilution test is an excellent test for investigating sBC-dependent biases.

The results of both these tests, show the presence of a negative bias in the AXSYM FT4 particularly in sera with low sBC levels. Such observations are consistent with recent data obtained during a College of American Pathologists (CAP) Ligand Assay Survey study (21). The investigators altered the protein bound T4 and sBC concentrations in their test panel, by filling three batches of vials with one pool of serum at a ratio of 0.5:1:2.0 (in the study the samples were coded K-95, K-96 and K-97, respectively), and then lyophilizing the dispensed sera. Survey participants were asked to reconstitute each of the three vials with the same volume of diluent that would reconstitute K-96 to its original concentration. The measured FT4 by AXSYM (CAP survey results) in the diluted serum pool (K95) was 11.1 pmol/l which was 23% lower than the concentration of 14.4 pmol/l seen in the concentrated serum (K97).

The mechanism of the sBC-dependent biases exhibited by the AXSYM FT4 assay is not known, but it is likely to be due to an excessive disturbance in the T4/binding-protein equilibrium produced by the assay reagents. The estimation of serum FT4 by immunoassay, irrespective of the methodology used will invariably disturb the normal equilibrium that exists between the PBT4 and sBC (2,3). This will come about as a result of the addition of the antibody and of dilution of the sample in the assay reagents, which may also contain T4 binders such as albumin or animal sera. These are often added to protect assays from interference from heterophilic antibodies (22). These additions will result in the establishment of a new equilibrium and a new "in vitro" FT4 concentration The concentration of this in vitro FT4 will be dictated by the "in vitro" bound T4 and unbound binding site concentrations, i.e. the in vitro FT4 will be equal to (PBT4+iBPT4)/(sBC + iBC), where iBPT4 is the T4 bound by the immunoassay reagents and iBC is the immunoassay binding capacity (which will be equal to the affinity multiplied by the concentration of the free immunoassay binding sites). From this formula, one can predict that a high iBC will cause biases, which 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 progressively lower results.

The results presented show that the AXSYM FT4 assay mimics the performance of an assay whose reagents contain significant concentrations of binding sites (i.e. iBC). The presence of such a binder in the AXSYM FT4 reagents was suggested by the fact that we measured an albumin concentration of 10 g/L in the assay reagent

used in the first incubation step. Inclusion of BSA in the reagents of FT4 assays has been argued (23) to be important in negating the influence of binding inhibitors which can be generated in vitro (e.g. non-esterified fatty acids which can be produced in sera of heparinized patients). However, whilst this notion is true, as Ekins (24) points out the inclusion of BSA will also "distort the effect of endogenous inhibitors on FT4 concentrations, causing assay results to be grossly unreliable when these are present". Inclusion of BSA in the assay reagents of the Vitros FT4 assay (which does not contain any BSA) induced gross alterations in the FT4 profile of the two serum samples tested. The FT4 value of the low sBC serum became progressively negatively biased, in parallel with increases in the BSA loading of the assay reagents, whereas, the FT4 value of normal sBC serum became positively biased. The FT4 profile obtained with the AXSYM FT4 method suggests that its reagents contain exogenous thyroid binding proteins that have a binding capacity which is equivalent to the binding capacity of a BSA concentration of >30g/l.

The present study highlights significant weaknesses in the design of the AXSYM FT4 assay, which result in significant inaccuracies when used to measure FT4 in patients whose serum T4 binding capacities are reduced. Furthermore, a possible mechanism for the methodological differences often described in the literature is suggested. This is likely to be due to variable disturbances of the normal serum T4/protein equilibrium brought about by the inclusion of variable amounts of BSA and/or other T4 binding proteins in the immunoassay reagents.

REFERENCES

1. Robbins J., Rall J.E. The interaction of thyroid hormones and protein in biological fluids. Recent Progress in Hormone Research 1957;13:161-208.

2. Ekins R. Measurement of free hormones in blood. Endocr Rev 1990;11:5-46.

3. Ekins R. The free hormone hypothesis and measurement of free hormones. Clin Chem 1992;38:1289-93.

4. Bayer M.F. Free thyroxine results are affected by albumin concentrations and non-thyroidal illness. Clin Chim Acta 1983;130:391-6.

5. Csako G., Zweig M.H., Benson C., Ruddel M. On the albumin dependence of measurements of thyroxin. I. Technical performance of seven methods. Clin Chem 1986;32:108-15.

6. Czako G., Zweig M.H., Benson C., Ruddel M. On the albumin-dependence of measurements of free thyroxin. II. Patients with non-thyroidal illnesses. Clin Chem 1987;33:87-92.

7. Csako G., Zweig M.H., Glickman J, Ruddel M., Kestner J. Direct and indirect techniques for free thyroxin compared in patients with non-thyroidal illness. II. Effect of prealbumin, albumin and thyroxin-binding globulin. Clin Chem 1989;35:1655-62.

8. Ooi G., Mahadevan M.S., Greenway D.C., Gertler S.Z. Evaluation of four commercially available assays for free thyroxin. Clin Chem 1988;34:2302-7.

9. Midgley J.E.M., Moon C.R., Wilkins T.A. Validity of analog free thyroxine immunoassays. Part II. [opinion]. Clin Chem 1987;33:2145-8.

10. Sapin R., Gasser F., Schlienger J.L. Free thyroxine in familial dysalbuminemic hyperthyroxinemia, as measured by five assays [Tech Brief]. Clin Chem 1988;34:598-9.

11. Christofides N.D., Sheehan C.P. Enhanced Chemiluminescence labeled-antibody immunoassay (Amerlite MAB) for free thyroxine: Design, development, and technical validation. Clin Chem 1995;41:17-23.

12. Christofides N.D., Wilkinson E., Stoddart M., Ray D.C., Beckett G.J. Assessment of serum thyroxine binding capacity-dependent biases in free thyroxine assays. Clin Chem 1999;45:520-525.

13. Nelson J.C., Weiss R.M., Wilcox R.B. Underestimates of serum free thyroxine (T4) concentrations by free T4 immunoassays. J Clin Endocrinol Metab 1994;79:76-9.

14. Nelson J.C., Nayak S.S., Wilcox R.B. Variable underestimates by serum free thyroxine (T4) immunoassays of free T4 concentrations in simple solutions. J Clin Endocrinol Metab 1994;79:1373-5.

15. Nelson J.C., Wilcox R.B. Analytical performance of free and total thyroxine assays. Clin Chem 1996;42:146-54.

16. Nelson J.C., Tomei R.T. Direct determination of free thyroxine in undiluted serum by equilibrium dialysis/radioimmunoassay. Clin Chem 1988;34:1737-44.

17. Cornbleet P.J., Gochman N. Incorrect least squares regression coefficients in method-comparison analysis. Clin Chem 1979;25:432-8.

18. Stockigt J.R. Guidelines for diagnosis and monitoring of thyroid disease: non-thyroidal illness. Clin Chem 1996; 42:188-192.

19. Becker D.V., Bigos S.T., Gaitan E., Morris J.C., Rallison M.L., Spencer C.A., et al. Optimal use of blood tests for assessment of thyroid function (Letter). JAMA 1993;269:2736.

20. Braverman L.E. Evaluation of thyroid status in patients with thyrotoxicosis. Clin Chem 1996;42:174-178.

21. Steele B.W., Witte D.L., Whitley R.J., Klee G.G., Chan D.W. The effects of modifying proficiency testing materials on thyroid function test results. Arch Pathol Lab Med, 1997; 121:1241-6.

22. Boscato L.M., Stuart M.C. Heterophilic antibodies: A problem for all immunoassays. Clin Chem 1988;34:27-33.

23. Wilkins T.A. Albumin in analog FT4 assay reagents; the facts (Letter). Clin Chem 1987;33:1293.

24. Ekins R. One-step, labeled-antibody assay for measuring free thyroxine. 1. Assay development and validation. Clin Chem 1992;38:2355-2357.