Serum triiodothyronine determination in clinical use

J. E. H. STAFFORD1, S. LEES, AND D. WATSON

From the Area Laboratory, King Edward VII Hospital, Windsor

SYNOPSIS Two radioimmunoassays for the determination of serum triiodothyronine (T3) were developed. The assay of T3 in unextracted serum had several advantages over the assay on extracted serum and was chosen for the routine determination of T3 in serum from 117 patients requiring assessment of their thyroid status.

In 53 subjects considered retrospectively not to have thyroid dysfunction nor to have been on steroid contraceptives or therapy, the pooled mean serum T3 concentration was 1.92 (actual range 0.88-2.62) nmol/l. A significant inverse relationship was observed between the serum T3 level and the age of the subject. Serum total T3 levels discriminate clearly between hypo-, eu- and hyper-thyroid patients and provide a rather more sensitive index of hyperthyroid function than total serum T4. In the face of normal serum T4, the T3 level was depressed in five patients with marked hypo-proteinaemia and elevated in two patients taking heroin.

The application of radioimmunological methods to the measurement of serum triiodothyronine (T3) in health and disease has revealed a previously unsuspected role for this hormone in the maintenance of the euthyroid state. Clinically, serum T3 estimations have been advocated for detecting early thyrotoxicosis (Hollander et al, 1971b) and have revealed a new variant of hyperthyroidism, 'T3-toxicosis', in which patients diagnosed unequivocally as thyrotoxic have normal serum thyroxine (T4) levels but increased concentrations of T3 (Sterling et al, 1970). In addition it is now clear that treatment of hyperthyroidism, by surgery or with drugs, leads to preferential serum T3 production (Bellabarba and Tremblay, 1972) as do low iodine diets (Hollander et al, 1971a).

The accuracy of methods based on protein-binding techniques for T3 analysis has been impaired by interference from in vivo T3-binding proteins, in particular thyroid hormone-binding globulin (TBG). When interference from this source has gone unrecognized high serum values for T3 have been reported (Gharib et al, 1970).

Several reagents capable of blocking plasma protein T3-binding sites have been reported and these include T4 (Chopra et al, 1971), tetrachlorothyronine (Mitsuma et al, 1971), diphenylhydantoiln (Lieblich and Utiger, 1972), salicylate (Larsen, 1971), and 8-anilino-naphthalene-sulphonic acid (Mitsuma et al, 1972). Each has drawbacks; either they are unstable in solution or they interfere with the antigen-antibody reaction (Hüfner and Hesch, 1973a). Recently, sodium ethylmercurithiosalicylate (Merthiolate, thiomersal) has been shown to inhibit the binding of T3 to thyroxine-binding proteins (Bellabarba and Tremblay, 1973), and this reagent has been incorporated into radioimmunoassays for triiodothyronine (Hufner and Hesch, 1973; Kirkegaard et al, 1974). In the presence of serum proteins, merthiolate does not influence the antibody-hapten reaction (Kanagasabapathy and Wellby, 1974).

This report describes a comparison of two radioimmunoassays for serum T3, the alcoholic extraction assay being used as a reference method for the assay on unextracted serum. Merthiolate was used to block the TBG binding sites. A preliminary clinical evaluation of the results on patients for whom routine in vitro thyroid function tests have been requested is presented.

Material and Methods

BARBITONE-ALBUMIN-MERTHIOLATE BUFFER
Barbitone buffer (0.1 mol/l) at pH 8.65 contained Merthiolate (sodium ethylmercurithiosalicylate, 5·00 mmol/l) and bovine albumin (5 g/l). All solutions were stored at 4°C unless otherwise stated.
LABELLED TRIODOTHYRONINE

High specific activity $^{125}$I-L-triiodothyronine (ca 400 mCi/mg) in 50% propylene glycol was purchased from Abbott Laboratories and further purification was found to be unnecessary. It was diluted to about 500 pmol/l with barbitone-albumin-Merthiolate buffer.

STANDARDS

L-3', 3, 5-triiodothyronine (B grade) was obtained from Calbiochem Ltd, and its molar extinction coefficient (Gemmill, 1955) in sodium hydroxide (40 mmol/l) was used as an index of purity. Further purification proved to be unnecessary. A stock standard solution (768 μmol/l) was prepared in acidified ethanol (10 ml HCl/l) and stored at −20°C. Standard T₃ was prepared by diluting the stock standard solution 125 times with sodium hydroxide (40 mmol/l). A 50 μl aliquot of this solution was diluted to 10 ml with T₃-free serum or with 67% ethanol, and these working standards were stored in small aliquots at −20°C.

Assay standards were prepared immediately before the assay by doubling dilutions of working standard in T₃-free serum or 67% ethanol to give from 768 to 24 fmol of T₃ per assay tube.

TRIODOTHYRONINE-FREE SERUM

Five grams of Norit A charcoal (Sigma) were added to 50 ml of pooled, human sera (visibly free from haemolysis and icterus) and gently stirred for one hour at room temperature. After centrifugation a further 4 g were added and the process was repeated. Finally the supernatant was passed through millipore filters (Sartorius Membrane filter SM 11306, 0.45 μm) and stored at −20°C. More than 99% of added $^{125}$I-T₃ was removed by this procedure.

ANTISERUM

The antiserum (No. 5303), raised in sheep against a triiodothyronine-albumin conjugate, was kindly donated by Professor R. P. Ekins (Institute of Nuclear Medicine, The Middlesex Hospital, London). Small volumes were stored at −20°C, thawed when required, and diluted to 1:1400 with barbitone-albumin-Merthiolate buffer.

CHARCOAL SUSPENSION

For the assay of T₃ in unextracted serum an 8 g/l suspension of Norit A charcoal in barbitone buffer was used. Assays carried out in 67% ethanol required the charcoal to be coated with Dextran T₇₀₀ (0.5 g/l) for the optimal separation of free and antibody-bound T₃.

PATIENTS AND PREPARATION OF SAMPLES

Thyroid function tests were requested on 117 patients seen by general practitioners. Blood was obtained by venepuncture from these outpatients and allowed to clot, and the serum was separated by centrifugation. The samples were stored at −20°C. In some experiments alcoholic extracts of serum were prepared by the addition of two volumes of ice-cold ethanol and spinning down the precipitated proteins. Recovery of added $^{125}$I-T₃ under these conditions was 100% and confirmed the results of Patel and Burger (1973). Duplicate 50 μl aliquots of the supernatant were used directly in the radioimmunoassay.

Thyroxine was measured by the Abbot Tetrasorb procedure controlled as previously described (Watson and Lees, 1973). Sera showing T₄ levels that were close to upper or lower normal limits were assayed for 'free thyroxine index' (FTI). The latter was calculated as total T₄ divided by the unsaturated thyroid hormone-binding globulin level, expressed as a fraction of a laboratory pooled serum standard and measured by the Thyopac-3 test (Radiochemical Centre).

Radioimmunoassay

To 50 μl of the unknowns, standards, and 'zero' standards were added 100 μl of $^{125}$I-T₃ solution and 100 μl of diluted antiserum. For the unknowns and 'zero' standard a similar set of tubes was set up containing buffer instead of antiserum (blanks). After mixing and incubation overnight at 4°C or for one hour at 37°C, the tubes were equilibrated in an ice bath. A 500 μl aliquot of a well-stirred charcoal suspension at 2°C was forcibly added to each tube with the aid of an automatic syringe pipette. After incubation for 20 minutes the tubes were centrifuged at 4000 rev/min for 10 minutes and returned to the ice bath. The supernatants were removed by suction and the charcoal pellets counted in an LKB Wallac 8000 gamma counter for at least 60 seconds (ie, about $10^4$ counts).

CALCULATIONS

All counts were automatically corrected for background.

Standards

The reciprocal of B, the fraction of T₃ bound by the antibody, was calculated by the formula:

$$\frac{1}{B} = \frac{F_0}{F_0 - F_u}$$

where B is the fraction bound, $F_0$ is the number of counts adsorbed by the charcoal in the blank, ie, in the absence of antiserum, and $F_u$ is the number of
counts adsorbed by the charcoal in each standard tube in the presence of antiserum. This was plotted against the amount of cold T₃ in each standard tube, and a reproducible linear relationship was found over the range 0-768 fmol T₃, corresponding to serum concentrations from zero to about 15 nmol/l.

**Unknowns**

Preliminary experiments indicated that the adsorption of free T₃ onto charcoal varied from serum to serum and it was found necessary to run a blank with each sample for accurate results. The reciprocal of the fraction bound was calculated as for the standards. The serum T₃ content was derived from the standard curve, corrected for any dilution and expressed as nmol/l.

**Statistics**

The mean, standard deviation, and correlation and regression coefficients were calculated according to Snedecor and Cochran (1956) on a programmable desk-top calculator.

**Results**

**SPECIFICITY**

Cross-reaction of the antisera with thyroxine (T₄), tetraiodothyroacetic acid, and triiodothyroacetic acid was less than 0.1% in each case. Doubling dilutions of the sera from thyrotoxic and euthyroid patients with T₃-free serum resulted in curves that did not differ significantly from the standard curve.

**RECOVERY EXPERIMENTS**

Recovery was determined by comparing the amount of T₃ detected by radioimmunoassay (y) with the known quantities (up to 600 fmol T₃) added to hypo-, eu-, and hyper-thyroid sera (x). For the assay on alcoholic extracts the relationship was given by the equation y = 1.02x + 4.72 (x and y units in fmol, r = 0.987, n = 16, and p < 0.001). For the unextracted serum assay the line was given by y = 1.02x - 1.08 (r = 0.980, n = 28, p < 0.001). When the same sample was analysed by both methods the amount found in the unextracted serum (y) was highly correlated with the level detected by the alcoholic extraction assay (x) and the relationship was described by the equation y = 1.05x - 0.02 (r = 0.973, n = 31, p < 0.001). The result confirms the ability of Merthiolate to block the T₃-binding sites of serum thyroxine-binding globulin. The incubation time could be reduced to one hour at 37°C without loss of accuracy or sensitivity. The estimate at 37°C (y) was related to the value at 4°C (x) by the equation y = 1.02x + 0.03 (r = 0.984, n = 18, p < 0.001).

**SENSITIVITY AND REPRODUCIBILITY**

For the assay on unextracted serum samples these statistics have been summarized in table I. The between-batch coefficient of variation for six assays was 8.6%. The coefficient of variation within-batch ranged from 14% in the hypothyroid group to 3% in the euthyroid group. Very similar figures were calculated for the alcoholic extraction assay with the exception that ethanol tended to depress the binding in the low dose region of the standard curve (intercept 1/B was 1.887 ± 0.092, n = 10). A direct result of this effect was a reduction in the assay sensitivity (smallest detectable dose 11 ± 6 fmol).

**NORMAL RANGE**

The normal range (mean ± 2 SD) of serum T₃ in 53 euthyroid outpatients was 1.92 ± 0.77 nmol/l (1.25 ± 0.50 µg/l). This agrees well with values found by most other investigators using radio-

<table>
<thead>
<tr>
<th>Standard Curve</th>
<th>Between-batch Variation</th>
<th>Within-batch Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept 1/B</td>
<td>1.582 ± 0.109*</td>
<td>1.582 ± 0.025</td>
</tr>
<tr>
<td>Inverse slope (pmol)</td>
<td>0.1754 ± 0.0274</td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9958 ± 0.0046</td>
<td></td>
</tr>
<tr>
<td>Smallest detectable dose (fmol)</td>
<td>2.9 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>50% of intercept (pmol)</td>
<td>0.293 ± 0.043</td>
<td>0.293 ± 0.018</td>
</tr>
<tr>
<td>Control serum (nmol/l)</td>
<td>95.32 ± 0.47</td>
<td>95.32 ± 0.68</td>
</tr>
<tr>
<td>Samples</td>
<td>2.048 ± 0.176</td>
<td>2.048 ± 0.156c</td>
</tr>
<tr>
<td>&lt; 0.75 nmol/l</td>
<td></td>
<td>0.512 ± 0.072d (13)*</td>
</tr>
<tr>
<td>&gt; 0.75 &lt; 2.25 nmol/l</td>
<td>2.081 ± 0.063 (78)</td>
<td></td>
</tr>
<tr>
<td>&gt; 2.25 nmol/l</td>
<td></td>
<td>4.075 ± 0.145 (35)</td>
</tr>
<tr>
<td>Charcoal adsorption</td>
<td>94.64 ± 0.92</td>
<td>94.64 ± 0.90</td>
</tr>
</tbody>
</table>

*Calculated from unweighted regression analysis, mean ± SD
SD of zero dose/slope at this point × √2 (Ekins et al, 1968)
Control serum at beginning and end of each run
*Calculated by the duplicates method (Snedecor and Cochran, 1956)
*Number of duplicates.
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immunoassay but different TGB blocking agents. A small but consistent fall in serum T₃ with increasing age was observed in the present study (figure) and confirms the earlier observations of Brunelle and Bohuon (1972) and Rubenstein et al (1973).

**Clinical Results**

Summarized in table II are the serum T₃ and T₄ levels in 117 unselected patients for whom in vitro thyroid function tests were requested. The biochemical data were compared with the retrospective assessment of a patient’s thyroid status, and the final assessment was based on the outcome of examination, consultant opinion, and all investigations.

The salient features are as follows. All 38 hyperthyroid patients had high serum T₃ concentrations although increased serum levels of thyroxine were observed in only 31 patients. Of the remainder with normal serum T₄ concentrations, four were clinically diagnosed as ‘early’ thyrotoxicosis, one as ‘recurrent’ thyrotoxicosis while two were initially correctly diagnosed as ‘T₃-toxicosis’. Four of the five neomercazole-treated thyrotoxics had increased serum T₃ levels but normal or low serum T₄ concentrations. Low serum levels of T₃ and T₄ were observed in all seven cases of overt myxoedema. One patient had a border-line low serum T₄ level but a normal serum T₃ concentration. The serum FTI was normal and the initial clinical diagnosis of hypothyroidism not supported. Low serum T₃ levels were found in all five patients with hypoproteinaemia from various causes. However, serum T₄ levels and the free thyroxine index were normal in every case. Two subjects, who admitted taking heroin, had raised serum T₃ concentrations (3-4 and 3-6 nmol/l) but normal serum T₄ levels (122 and 133 nmol/l) and normal FTI (7-5 and 8-4).

**Discussion**

A radioimmunoassay for the direct measurement of T₃ in serum has been described and its performance compared with an alcoholic extraction assay. For routine use the direct assay was preferred, primarily because the time for antibody incubation could be reduced to one hour at 37°C and fewer pipetting steps were involved. Thus results could be obtained on the same working day with fewer potential sources

**Table II**  
<table>
<thead>
<tr>
<th>Serum T₃ and T₄ levels in 117 patients for whom thyroid function tests were required</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Final Assessment</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Hypothyroid</td>
</tr>
<tr>
<td>Hypoproteinaemic</td>
</tr>
<tr>
<td>T₄-treated hypothyroids</td>
</tr>
<tr>
<td>Euthyroid</td>
</tr>
<tr>
<td>Pregnant euthyroid</td>
</tr>
<tr>
<td>Clinically euthyroid heroin users</td>
</tr>
<tr>
<td>Hyperthyroid</td>
</tr>
<tr>
<td>Neomercazole treated hyperthyroid</td>
</tr>
<tr>
<td>Thyroidectomized hyperthyroid on T₄ replacement</td>
</tr>
</tbody>
</table>

*Less than 2SD below the mean. Normal range for T₄ is 73 to 163 nmol/l
**Greater than 2SD above the mean
*Total number of cases in each group
*Number of cases in each subgroup
*Same patient.
of error. In terms of accuracy the direct assay over-
estimated the T₃ content on average by some 5%.
This value agrees well with the 95% efficacy of
Merthiolate in displacing T₃ from TBG binding sites
reported by Hüfner and Hesch (1973a) and

A disturbing feature of the radioimmunoassay
measurement of T₃ in unextracted serum is the oc-
currence of low values in patients with hypoprotein-
aemia as a result of nephrotic syndrome, coeliac
disease or senile malabsorption. Low serum T₃
levels have also been detected in kwashiorkor. How-
ever, in the latter case this observation appears to be
due to a methodological artefact, since no low T₃
values were observed when sufficient T₃-free normal
serum was added to the immunoassay tube to make
the serum protein level comparable to normal
serum samples (van der Westhuyzen, 1973). Thus
before any reliance can be placed on serum T₃ data
from hypoproteinaemic patients it is essential that
the immunoassay system be shown to be unaffected
by large changes in serum protein levels. Further
work is needed to elucidate this phenomenon. How-
ever, normal patient to patient variation in total
serum protein content does not appear to affect the
accuracy of the method significantly, and in practice
it is the high serum T₃ values that are of clinical
importance.

Published ranges for euthyroid levels of serum T₃
as determined by radioimmunoassay vary consider-
ably, although workers have agreed that the mean
lies between 1·5 and 2·3 nmol/l with a standard de-
viation of some 0·3 to 0·5 nmol/l. This inter-
laboratory variation could be due to methodological
differences since the apparent serum T₃ concentra-
tion depends to some extent upon the blocking
agent used (Hüfner and Hesch, 1973a), but diet and
age of the control group may also be important.
Provided that the correct normal ranges are estab-
lished for each area laboratory, small dietary differ-
ences should not affect clinical conclusions based
on the radioimmunoassay data. However, the age
distribution of the population studied might do so.

If the normal population confidence limits derived
from a least squares regression analysis of serum T₃
level on patient’s age are compared with those of the
poled data then areas can be identified where
younger patients may be misclassified as early thyro-
toxicosis and older patients wrongly considered to be
euthyroid (figure). However, more data are re-
quired before the diagnostic value of this refinement
of the normal range can be assessed. No age-
dependent changes in serum T₄ concentration were
observed in the present study. In a much larger group
of euthyroid patients, a small but significant de-
crease in serum T₄ levels was observed in patients over
65 years of age (Herrmann et al, 1974). The authors
concluded that reduced protein-binding of thyroid
hormones, particularly T₃, could be excluded as the
cause of low serum T₃ and T₄ concentrations ob-
served in aged patients.

At the present time the usefulness of serum T₃
measurements in the routine diagnosis of thyroid
disease is restricted to two well-defined areas. Where
the clinical pattern indicates hyperthyroidism, but
serum T₄ level and the FTI are normal, measurement
of serum T₃ concentrations can confirm ‘T₃-toxi-
cosis’, and seven such patients were identified in the
present series. Of these seven patients, four were
diagnosed as ‘early thyrotoxicosis’. The ability to
provide biochemical support for this diagnosis is of
great value and suggests that all patients for whom
serum T₄ estimations have been requested should
also be screened for serum T₃. An increased serum
T₃ level relative to the T₄ concentration has been
observed during treatment of hyperthyroidism
(Bellabarba and Tremblay, 1972) and this occurred
in four patients in the present study. In these cases
measurement of serum T₃ levels helps to define the
thyroid status.

Not all cases of increased serum levels of T₃,
however, appear to be associated with clinical evi-
dence of hyperthyroidism. In this study two clinically
euthyroid heroin addicts had high levels of serum
T₃ but normal T₄ concentrations. Whether this
reflects the pituitary-thyroid relationship seen in
Pendred’s syndrome (deafness and non-iodine-
deficiency goitre) is not known, but Gomez-Pan et
al (1974) recently reported such a patient with raised
serum T₃ concentrations and normal levels of serum
T₄. Interestingly an increased TSH response to thyro-
tropin releasing hormone (TRH) was observed in
this patient. Studies in mice have shown that
codeine, which has a chemical structure very
similar to that of heroin, enhances the TSH response
to TRH (Redding et al, 1966). While caution must
be exercised in drawing inferences from animal
studies and applying them to the human it remains a
possibility that heroin was having a similar effect in
the patients addicted to this drug. However, in a
series of clinically euthyroid heroin addicts studied
by Webster et al (1973) an increased mean serum T₄
level was observed as a result of an elevated serum
TBG binding capacity. Serum T₃ levels were not
measured.

In conclusion, a preliminary clinical evaluation of
serum T₃ estimations in routine diagnostic use sug-
gests that measurement of serum T₃ levels would
provide more information than T₄ and should be re-
quired in all cases of suspected hyperthyroidism and
in patients undergoing therapy for thyroid dis-
orders.
References


Larsen, P. R. (1971). Inhibition of triiodothyronine (T3) binding to thyroxine-binding globulin by sodium salicylate and its application to immunoassay of T3 in human serum. Metabolism, 20, 976-980.


