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 (T_3) were developed. The assay of T_3 in unextracted serum had several advantages over the assay on extracted serum and was chosen for the routine determination of T_3 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 T_3 concentration was 1.92 (actual range 0.88-2.62) nmol/l. A significant inverse relationship was observed between the serum T_3 level and the age of the subject. Serum total T_3 levels discriminate clearly between hypo-, eu- and hyperthyroid patients and provide a rather more sensitive index of hyperthyroid function than total serum T_4 . In the face of normal serum T_4 , the T_3 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 (T_3) in health and disease has revealed a previously unsuspected role for this hormone in the maintenance of the euthyroid state. Clinically, serum T_3 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 thyrotoxicosis have normal serum thyroxine (T_4) levels but increased concentrations of T_3 (Sterling *et al*, 1970). In addition it is now clear that treatment of hyperthyroidism, by surgery or with drugs, leads to preferential serum T_3 production (Bellabarba and Tremblay, 1972) as do low iodine diets (Hollander *et al*, 1971a).

The accuracy of methods based on protein-binding techniques for T_3 analysis has been impaired by interference from *in vivo* T_3 -binding proteins, in particular thyroid hormone-binding globulin (TBG). When interference from this source has gone unrecognized high serum values for T_3 have been reported (Gharib *et al*, 1970).

Several reagents capable of blocking plasma protein T_3 -binding sites have been reported and these include T_4 (Chopra *et al*, 1971), tetrachlorothyronine

(Mitsuma *et al*, 1971), diphenylhydantoin (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 T_3 to thyroxine-binding proteins (Bellabarba and Tremblay, 1973), and this reagent has been incorporated into radioimmunoassays for triiodothyronine (Hüfner and Hesch, 1973b; 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 T_3 , 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.

¹Present address: Pharmacological Biochemistry, G. D. Searle & Co Ltd, Lane End Road, High Wycombe, Bucks HP12 4HL

LABELLED TRIIODOTHYRONINE

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 $\mu\text{mol/l}$) was prepared in acidified ethanol (10 ml HCl/l) and stored at -20°C . Standard T_3 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 T_3 -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 T_3 -free serum or 67% ethanol to give from 768 to 24 fmol of T_3 per assay tube.

TRIIODOTHYRONINE-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 - T_3 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 T_3 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 T_{70} (0.5 g/l) for the optimal separation of free and antibody-bound T_3 .

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 - T_3 under these conditions was 100% and confirms 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 T_4 levels that were close to upper or lower normal limits were assayed for 'free thyroxine index' (FTI). The latter was calculated as total T_4 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 - T_3 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 T_3 bound by the antibody, was calculated by the formula:

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

where B is the fraction bound, F_o 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 T_3 in each standard tube, and a reproducible linear relationship was found over the range 0-768 fmol T_3 , corresponding to serum concentrations from zero to about 15 nmol/l.

Unknowns

Preliminary experiments indicated that the adsorption of free T_3 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 T_3 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 (T_4), tetraiodothyroacetic acid, and triiodothyroacetic acid was less than 0.1% in each case. Doubling dilutions of the sera from thyrotoxic and euthyroid patients with T_3 -free serum resulted in curves that did not differ significantly from the standard curve.

RECOVERY EXPERIMENTS

Recovery was determined by comparing the amount of T_3 detected by radioimmunoassay (y) with the known quantities (up to 600 fmol T_3) 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 T_3 -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 T_3 in 53 euthyroid outpatients was 1.92 ± 0.77 nmol/l (1.25 ± 0.50 $\mu\text{g/l}$). This agrees well with values found by most other investigators using radio-

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

Table I Summary of radioimmunoassay characteristics

^aCalculated from unweighted regression analysis, mean $\pm$ SD

^bSD of zero dose/slope at this point $\times \sqrt{2}$ (Ekins *et al*, 1968)

^cControl serum at beginning and end of each run

^dCalculated by the duplicates method (Snedecor and Cochran, 1956)

^eNumber of duplicates.

Figure A significant inverse correlation was observed between serum T_3 levels and age: $y = 2.34 - 0.0081x$ ($n = 53$, $r = 0.393$, $p < 0.01$). The 95% confidence limits for the regression equation are shown by the solid lines. The dashed lines delineate the 95% confidence limits for the pooled data.

immunoassay but different TGB blocking agents. A small but consistent fall in serum T_3 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 T_3 and T_4 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 T_3 concentrations although increased serum levels of thyroxine were observed in only 31 patients. Of the remainder with normal serum T_4 concentrations, four were clinically diagnosed as 'early' thyrotoxicosis, one as 'recurrent' thyrotoxicosis while two were initially correctly diagnosed as ' T_3 -toxicosis'. Four of the five neomercazole-treated thyrotoxics had increased serum T_3 levels but normal or low serum T_4 concentrations. Low serum levels of T_3 and T_4 were observed in all seven cases of overt myxoedema. One patient had a border-line low serum T_4 level but a normal serum T_3 concentration. The serum FTI was normal and the initial clinical diagnosis of hypothyroidism not supported. Low serum T_3 levels were found in all five patients with hypoproteinaemia from various causes. However, serum T_4 levels and the free thyroxine index were normal in every case. Two subjects, who admitted taking heroin, had raised serum T_3 concentrations (3.4 and 3.6 nmol/l) but normal serum T_4 levels (122 and 133 nmol/l) and normal FTI (7.5 and 8.4).

Discussion

A radioimmunoassay for the direct measurement of T_3 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

Final Assessment		T_3			T_4		
		Low ^a	Normal	High ^b	Low ^a	Normal	High ^b
Hypothyroid	(8) ^c	7 ^d	1 ^e	0	7	1 ^e	0
Hypoproteinaemic	(5)	5	0	0	0	5	0
T_4 -treated hypothyroids	(2)	0	1	1	0	0	2
Euthyroid	(53)	2	51	0	0	51	2
Pregnant euthyroid	(3)	0	0	3	0	0	3
Clinically euthyroid heroin users	(2)	0	0	2	0	2	0
Hyperthyroid	(38)	0	0	38	0	7	31
Neomercazole treated hyperthyroid	(5)	0	1	4	2	3	0
Thyroidectomized hyperthyroid on T_4 replacement	(1)	0	0	1	0	0	1

Table II Serum T_3 and T_4 levels in 117 patients for whom thyroid function tests were required

^aLess than 2SD below the mean. Normal range for T_4 is 73 to 163 nmol/l

^bGreater than 2SD above the mean

^cTotal number of cases in each group

^dNumber of cases in each subgroup

^eSame patient.

of error. In terms of accuracy the direct assay overestimated the T_3 content on average by some 5%. This value agrees well with the 95% efficacy of Merthiolate in displacing T_3 from TBG binding sites reported by Hüfner and Hesch (1973a) and Kirkegaard *et al* (1974).

A disturbing feature of the radioimmunological measurement of T_3 in unextracted serum is the occurrence of low values in patients with hypoproteinaemia as a result of nephrotic syndrome, coeliac disease or senile malabsorption. Low serum T_3 levels have also been detected in kwashiorkor. However, in the latter case this observation appears to be due to a methodological artefact, since no low T_3 values were observed when sufficient T_3 -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 T_3 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. However, 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 T_3 values that are of clinical importance.

Published ranges for euthyroid levels of serum T_3 as determined by radioimmunoassay vary considerably, although workers have agreed that the mean lies between 1.5 and 2.3 nmol/l with a standard deviation of some 0.3 to 0.5 nmol/l. This inter-laboratory variation could be due to methodological differences since the apparent serum T_3 concentration 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 established for each area laboratory, small dietary differences 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 T_3 level on patient's age are compared with those of the pooled data then areas can be identified where younger patients may be misclassified as early thyrotoxicosis and older patients wrongly considered to be euthyroid (figure). However, more data are required before the diagnostic value of this refinement of the normal range can be assessed. No age-dependent changes in serum T_4 concentration were observed in the present study. In a much larger group of euthyroid patients, a small but significant decrease in serum T_4 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 T_3 , could be excluded as the cause of low serum T_3 and T_4 concentrations observed in aged patients.

At the present time the usefulness of serum T_3 measurements in the routine diagnosis of thyroid disease is restricted to two well-defined areas. Where the clinical pattern indicates hyperthyroidism, but serum T_4 level and the FTI are normal, measurement of serum T_3 concentrations can confirm ' T_3 -toxicosis', 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 T_4 estimations have been requested should also be screened for serum T_3 . An increased serum T_3 level relative to the T_4 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 T_3 levels helps to define thyroid status.

Not all cases of increased serum levels of T_3 , however, appear to be associated with clinical evidence of hyperthyroidism. In this study two clinically euthyroid heroin addicts had high levels of serum T_3 but normal T_4 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 T_3 concentrations and normal levels of serum T_4 . Interestingly an increased TSH response to thyrotropin 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 T_4 level was observed as a result of an elevated serum TBG binding capacity. Serum T_3 levels were not measured.

In conclusion, a preliminary clinical evaluation of serum T_3 estimations in routine diagnostic use suggests that measurement of serum T_3 levels would provide more information than T_4 and should be requested in all cases of suspected hyperthyroidism and in patients undergoing therapy for thyroid disorders.

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