A comparative study of serum total thyroxine estimation on unextracted serum by radioimmunoassay and by competitive protein binding

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SYNOPSIS A rapid and precise radioimmunoassay (RIA) for serum total thyroxine (T_4) on as little as 1-10 μ l of unextracted serum is described. Results in hypothyroidism (overt and borderline), in euthyroid subjects, in pregnant and oestrogen-medicated subjects, and in hyperthyroidism (overt and borderline) are compared with the results on the same sera by an established competitive protein-binding technique (Ames' Tetralute) on unextracted serum from a different laboratory. The correlation between the two methods was excellent ($r = 0.94$) and no significant difference between overall results or results in any subgroup was found. In the hypothyroid range the radioimmunoassay appeared to measure total T_4 reliably in sera containing only 1.3 or 2.6 nmol/l. Both methods predicted the clinical outcome in borderline hypothyroidism and borderline hyperthyroidism equally well and both gave normal results in T_3 -toxicosis. It is concluded that both techniques reliably measure total T_4 . RIA appears to have advantages of sensitivity and precision (especially in the hypothyroid range), of simplicity, and of low cost.

The determination of serum total thyroxine (T_4) is still the most widely used single measurement employed in the clinical assessment of thyroid function (Chopra, 1972). Until recently the methods available largely relied on prior extraction of serum followed by competitive protein-binding (CPB) assay (Murphy, 1965) though CPB analyses on unextracted serum have recently been described (Bauer *et al*, 1970; Braverman *et al*, 1971). A new approach has been the use of radioimmunoassay (RIA) on unextracted serum for the measurement of total serum T_4 and such techniques have been described by several authors (Chopra, 1972; Mitsuma *et al*, 1972; Beckers *et al*, 1973). However, discrepancies have been observed between the values of total T_4 found by RIA and by CPB techniques. Chopra (1972) found that values in euthyroid and hyperthyroid subjects were higher by RIA; the difference was not thought to be due simply to losses in the extraction procedure employed in the CPB technique and was attributed to the presence in some sera of T_4 which was linked by covalent bonds to serum proteins and not extracted by butanol/ethanol.

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Similarly, Beckers *et al* (1973) found higher values in hyperthyroid subjects but not in euthyroid subjects or in pregnant women, and Ratcliffe *et al* (1974) found higher values by RIA in hyperthyroid subjects. However, Mitsuma *et al* (1972), in a comparison of results from hypothyroid, euthyroid, and hyperthyroid subjects, found excellent agreement (coefficient of correlation 0.98).

This paper compares total T_4 estimation on unextracted serum by RIA and CPB techniques performed in two different laboratories.

Methods

Total thyroxine (T_4) was measured by RIA on unextracted serum using the method of Mitsuma *et al* (1972) modified as stated below.

^{125}I - T_4 (S.A. 25-50 mCi/mg) was obtained from the Radiochemical Centre, Amersham instead of from Abbott Laboratories, Illinois; 25 pg was used as label in the assay without further purification and counting time adjusted to give 20 000 counts above background in the zero control tubes. Mitsuma *et al* (1972) used 50 pg of ^{131}I - T_4 .

8-Anilino, 1-naphthalene sulphonic acid (ANS), Eastman Kodak Corp, was used to block T_4 binding to TBG in a dose of 200 μg per assay tube (instead of 175 μg) and was included both in standards and in diluted unknown sera. The assay was conducted in barbitone buffer 0.08 mol/l to block T_4 binding to thyroxine-binding pre-albumin (TBPA). The use of human serum freed from T_4 and tri-iodothyronine (T_3) by charcoal stripping (Mitsuma *et al*, 1972) was obviated by using high dilutions of serum in buffer (100 μl of 1/10-1/100 dilution of test serum routinely, but even higher dilutions for some hyperthyroid sera). At these high dilutions the slope of the curve formed by the serial dilution of a hyperthyroid serum (serum T_4 concentration 282 nmol/l) followed exactly the standard curve constructed in buffer (fig 1). Figure 2 shows a comparison of standard curves made up in buffer and in three dilutions of T_4 -free serum; at dilutions of serum less than 1:10 the standard curve was displaced from the standard curve constructed in buffer but at serum dilutions of 1/10 or higher the curve was parallel. Owing to the sensitivity of the method specimens from hypothyroid, euthyroid, or hyperthyroid subjects could be appropriately diluted so that the percentage bound fraction fell on the steep portion of the standard curve (area of greatest precision), and even at the lowest dilution (1/10) non-specific interference by serum is avoided. A hyperthyroid serum assayed in multiple dilutions from 1/25 (4 μl of serum) to 1/800 (0.125 μl of serum) showed a value of 282 nmol/l with a coefficient of variation of 9.6%. A hypothyroid serum assayed in multiple dilutions from 1/10 (10 μl of serum) to 1/25 (4 μl of serum) had a mean value of 4.7 nmol/l with a coefficient of variation 14.9%.

Non-radioactive standards of T_4 (12.5-500 pg/tube

Fig 1 The curve formed by serial dilution of a hyperthyroid serum (T_4 content 282 nmol/l) assayed at 1/25 to 1/800 was parallel to the standard curve constructed without T_4 -free serum.

Fig 2 Standard curves constructed in the presence of T_4 -free serum (T_4 FS) at dilutions greater than 1:10 were parallel with a standard curve not containing T_4 FS. At greater serum concentrations the standard curve was displaced.

routinely) were derived by serial dilution in buffer of T_4 (10 mg of free acid) from Sigma Chem Co., dissolved initially in 0.13 M NaOH in 70% ethanol (20 ml) and stored at -20°C . This stock solution was renewed monthly. Standards were made up fresh for each assay by serial dilution from the stock solution. Mitsuma *et al* (1972) used standards of T_4 of 0.1-5 ng per assay tube.

Antiserum to T_4 was produced in rabbits by injection of T_4 conjugated to bovine serum albumin prepared by the method of Gharib *et al* (1971). The antiserum showed 8% cross-reactivity with T_3 as assessed by comparison of the doses of T_4 and T_3 required to inhibit binding by 50% from the zero control. Even if serum from a hyperthyroid patient with a serum T_3 concentration of 6.0 ng/ml were used at a dilution of only 1/10, which would be unusual, the T_3 content (60 pg) would cause a negligible depression of the percentage bound fraction. Cross-reactivity with mono- and di-iodotyrosine was not significant. The assay regularly detected 12.5 pg T_4 equivalent to a serum concentration of 1.6 nmol/l.

ASSAY PROCEDURE

- 100 μl ^{125}I - T_4 (25 pg)
- 100 μl antiserum at 1/100 initial dilution
- 100 μl diluted serum sample or T_4 standard
- 200 μl ANS (200 μg).

The assay was run in barbitone buffer. Incubation was at 37°C for 1½ hours in a shaking water-bath.

Separation of bound from free T_4 was achieved by a dextran-charcoal system essentially as described by Mitsuma *et al* (1972). The percentage bound fraction was counted overnight on a Packard Auto-gamma counter. The assay takes three hours apart from the counting and results are available the following morning.

RECOVERY

Recoveries were calculated by the addition of T_4 to euthyroid and hyperthyroid sera of which the basal T_4 content was also measured. For final T_4 concentrations of 87.5–1588 nmol/l the mean recovery was 96% (range 91–105%) in 10 experiments. Recoveries were also calculated in two experiments, by the addition of T_4 to T_4 -free serum and found to be 95 and 102%.

PRECISION

For hypothyroid, euthyroid, and hyperthyroid sera the within-batch SD of duplicate analyses was 2.7, 5.3, and 14.8 nmol/l, representing coefficients of variation of 19.8, 5.3, and 7.5% respectively. The between-batch SD was 2.2, 5.2, and 17.5 nmol/l for hypothyroid, euthyroid and hyperthyroid sera respectively analysed in duplicate on consecutive assays. A quality control serum analysed over six assays had a coefficient of variation of 9% (mean value 295 nmol/l).

COMPETITIVE PROTEIN-BINDING

Total T_4 by CPB was measured by the Ames Tetralute Sephadex column method (Howorth and McKerron, 1974) which included the modifications described by Gyde *et al* (1973). The coefficient of variation within-batch was 9.8% and between-batch was 12.7% (52 and 38 duplicate analyses respectively). Separate aliquots of the specimens were analysed by the two methods by separate laboratories over a period of several months, being kept deep frozen at -20°C pending analysis.

Subjects

Fourteen of the hypothyroid subjects were overt clinical cases of myxoedema and all have shown a good response to subsequent treatment with T_4 . Four subjects had dubious clinical evidence of hypothyroidism but all had an elevated serum TSH concentration; on follow-up three patients developed overt hypothyroidism responsive to treatment and one remained unchanged for a prolonged period. Of 30 euthyroid subjects, 16 were normal healthy volunteers and 14 were clinically euthyroid hospital outpatients.

Twelve subjects were taking contraceptive

preparations containing 50 μg of oestrogen and another 12 were pregnant.

Of the 18 hyperthyroid subjects, 14 were overt clinical cases of thyrotoxicosis and four cases were considered to be clinically borderline. The diagnosis in all cases was established by response to anti-thyroid medication. All 18 patients had an elevated serum T_3 concentration measured by RIA on unextracted serum (Marsden and McKerron, 1975).

Results

Excellent overall agreement was found between the two methods with a correlation coefficient of 0.94 (see fig 3).

The mean and SD of total T_4 by each method in each subgroup of subjects is shown in the table. None of these differences is significant and there was no overall significant difference ($P > 0.05$).

COMPARISON WITH CLINICAL DATA

On the assumption that the normal range of 51.5–141.6 nmol/l for total T_4 established by the CPB technique (Howorth and McKerron, 1974) applies to both methods, the two methods were compared for their ability to predict clinical thyroid status.

HYPOTHYROIDISM

In overt hypothyroidism RIA gave 13/14 and CPB 14/14 results below 51.5 nmol/l. In borderline hypothyroidism each method gave the same results, three within the normal range and one below; one patient out of the four remained clinically unchanged for two years despite an elevated serum

Fig 3 Correlation of serum T_4 concentrations (nmol/l) by radioimmunoassay (T_4 RIA) and competitive protein binding (T_4 CPB) in different groups of subjects. The normal range is indicated by the interrupted lines.

	Overt Hypothyroid (14)	Borderline Hypothyroid (4)	Euthyroid (30)	Oral Oestrogens or Pregnancy (24)	Hyperthyroid (18)	Totals (90)
RIA	7.7 $\pm$ 9.0	51.5 $\pm$ 24.5	81.1 $\pm$ 14.2	114.5 $\pm$ 24.5	182.8 $\pm$ 55.3	97 $\pm$ 62
CPB	14.2 $\pm$ 15.4	52.8 $\pm$ 18.0	88.8 $\pm$ 20.6	115.8 $\pm$ 20.6	195.6 $\pm$ 55.3	104 $\pm$ 64

Table Comparison of the mean and SD of total serum T₄ measured by radioimmunoassay (RIA) and competitive protein binding (CPB) techniques on unextracted serum

Figures in parentheses indicate numbers of subjects; units in nmol/l

TSH concentration (T₄ normal by both methods); the other three patients became overtly hypothyroid despite normal T₄ concentrations in two of them by both methods.

Euthyroid subjects

Each method gave only one value below 51.5 nmol/l, otherwise all results were within the normal range.

Oestrogens

All 12 subjects on oral contraceptives fell within the normal range by RIA, 11 of them by CPB. Of 12 pregnant subjects, three were above the normal range by RIA and one by CPB.

Hyperthyroid subjects

For the four clinically borderline subjects RIA gave two results within the normal range and CPB gave three. In one of the clinically overt cases the CPB method gave a T₄ slightly above and RIA slightly below the upper limit of normal; in the remainder results by both methods were elevated above normal.

Discussion

The radioimmunoassay described here is precise, simple, and cheap. Consumables and reagents cost approximately £360 per annum, to which may be added the estimated labour cost of £500 per annum (one-quarter of a technician's salary). The costing for the competitive protein-binding technique on the same basis was £360 for consumables and reagents, and £1000 for labour provided Sephadex columns are reutilized. Both methods are considerably cheaper than 'kits' commercially available. The annual sample load for the CPB method at the time of costing was 3000 samples and the RIA accommodates this number or more on the cost analysis given.

A new methodological feature is the non-dependence on T₄-free human serum which other assays have employed (Mitsuma *et al*, 1972). This is made possible by measurement of T₄ in serum at high dilution. Any tendency for a serum effect as described for the T₃ immunoassay (Mitsuma *et al*,

1972) on this assay would result in RIA concentrations appearing falsely higher than values by competitive protein-binding, which is clearly not the case.

A further feature of the RIA resulting from the sensitivity of the method is the opportunity to dilute the specimen so that the T₄ content falls on the steep (precise) portion of the standard curve. The sensitivity and precision are greater than those reported for most CPB techniques and are of particular value in the measurement of hypothyroid specimens. The use of such small volumes of serum as described here may well be of advantage in paediatrics or other circumstances where it is difficult to obtain blood by routine venepuncture, or in experimental work employing small animals.

Other workers have described higher values for total T₄ by RIA than by CPB as mentioned in the introduction. The CPB techniques employed have largely relied on a prior extraction of serum and it has recently been shown that the recovery of T₄ may fall off progressively with higher total serum T₄ concentrations when using such a technique (Hehrmann and Schneider, 1974). The very close agreement in total T₄ concentrations and in prediction of clinical status found over a wide range of subjects by the two assays described here, which are both performed on unextracted serum, suggests that the previously described difference between the two techniques may well be an artefact of extraction procedures, particularly as identity of results has been described when T₄ is measured by RIA and CPB on extracts of serum (Chopra, 1972) or when T₄ is measured by direct RIA and by RIA on extracts of serum after correction with an extraction factor (Hehrmann and Schneider, 1974). Excellent recovery is reported with this assay at extremely high total T₄ concentrations (1588 nmol/l). Our findings would therefore suggest that both RIA and CPB techniques on unextracted serum reliably quantitate total serum T₄.

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