Introduction of a rapid, simple radioimmunoassay and quality control scheme for thyroxine

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SYNOPSIS A simple radioimmunoassay has been developed for service purposes to determine serum total thyroxine levels. Only three additions are required, of standard or sample, labelled thyroxine and antibody in polyethylene glycol. After 2 hours' incubation at room temperature the antibody-bound and free fractions are separated by centrifugation. Serum total thyroxine levels were measured in 195 euthyroid subjects and it was established that normal values lay within the range 57 to 155 nmol/l. Serial blood samples taken over a 24-hour period, from 11 subjects, indicated that there was no circadian rhythm so that samples for total thyroxine assay can be taken at any time of the day. Similar results were obtained using serum or plasma. Satisfactory results were obtained for three quality control sera when measured by seven different laboratories using this method.

Until recently the determination of protein-bound iodine (PBI) was the single most widely used *in-vitro* test for the evaluation of thyroid function; however, its diagnostic accuracy is severely impaired because many samples are contaminated by iodine. Murphy and Pattee (1964) developed a protein-binding assay for thyroxine (T₄) which was unaffected by either endogenous or exogenous iodine. Because of difficulties in raising suitable antibodies, the development of more specific radioimmunoassays was slow. In 1971, Chopra and his colleagues succeeded in raising antibodies to T₄ by immunizing rabbits with thyroglobulin, and since then other workers have produced more specific antibodies with higher titres by immunizing rabbits and sheep with T₄:serum albumin conjugates (Mitsuma *et al*, 1972; Corcoran *et al*, 1973).

Several radioimmunoassays for T₄ have now been developed employing a variety of compounds to block endogenous thyroxine-binding globulin (of which the most common are 8-anilino 1-naphthalene sulphonic acid and merthiolate), barbital buffer to block binding to endogenous pre-albumin, and several different techniques to separate the antibody-bound and free fractions. A comparison of polyethylene glycol (PEG), charcoal, antibody bound to

solid phase supports, and ion exchange resin (Ratcliffe *et al*, 1974a) showed that PEG gave the lowest 'between batch' coefficient of variation, was inexpensive (cost per tube 0.03p), and, unlike charcoal, could be added at the beginning of the assay and was not time dependent.

The present paper summarizes our experience with a service radioimmunoassay for T₄ based on the use of PEG for separation and phosphate rather than barbital buffer, as the former was employed for the other service assays. The results of a quality control scheme involving seven different laboratories which employ the same reagents and assay protocol are also reported.

Material and Methods

BUFFERS

0.05M phosphate, pH 7.4

0.05M barbital, pH 8.6

T₄ FREE SERUM

T₄ was extracted by the addition of 10 g of dry, unwashed Norit OL charcoal (Hopkins and Williams) to 100 ml of pooled serum and mixing overnight by vertical rotation at room temperature. The charcoal and adsorbed T₄ were removed by centrifugation and filtration through millipore filters. The addition of a trace amount of ¹²⁵I-T₄ showed that 98-100% was removed.

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STANDARD SOLUTIONS

T₄, in the free acid form (obtained from Sigma), was dissolved in 0.02M NaOH and diluted in T₄ free serum to give solutions containing 10 to 400 nmol/l.

ANS/¹²⁵I-T₄

ANS (obtained from Sigma) was dissolved in buffer to give a solution containing 8 mg/ml, to which was added ¹²⁵I-T₄ (obtained from the Radiochemical Centre, Amersham) at a concentration of 10 ng/ml.

POLYETHYLENE GLYCOL 6000 (PEG)

PEG (obtained from British Drug Houses) was dissolved in buffer to give a solution containing 200 g/l.

ANTI-T₄ SERUM

An antiserum was raised by Dr T. G. Merrett* in a sheep by immunization with a T₄:BSA conjugate, which cross-reacted with triiodothyronine only to the extent of 1.3%.

ANTI-T₄/PEG

Antiserum was added to 20% PEG during vigorous mixing on a magnetic stirrer and was allowed to mix for a further 10 minutes before addition to the incubation tubes. This ensured an even suspension.

THYROXINE RADIOIMMUNOASSAY (T₄ RIA)

Reagents were added to LP3 tubes in the order and volumes summarized below:

Serum or standard T₄ 100 μ l

¹²⁵I-T₄/ANS 100 μ l

(1 ng) (800 μ g)

Anti-T₄ serum in 20% PEG 500 μ l (to give a final antiserum dilution of 1:2500)

In addition three sets of tubes were prepared to measure:

(a) the maximum binding of ¹²⁵I-T₄ (B₀) in which unlabelled T₄ was replaced by T₄ free serum;

(b) the blank (B_b) in which unlabelled T₄ was replaced by T₄ free serum and the antibody/PEG solution by 20% PEG, and

(c) the total radioactivity (T) in which ¹²⁵I-T₄ only was added. The tubes were vortexed and incubated at room temperature for 2 hours. Antibody-bound and free fractions were separated by centrifugation for 15 minutes at 700 g and aspiration of the supernatant fluid. The precipitates, which contained the bound fraction, were counted in an automatic gamma counter.

In this laboratory, standards and serum samples were dispensed with an Ependorf pipette, ANS/

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¹²⁵I-T₄ using a Hamilton syringe, and antibody/PEG with a repeating syringe.

CALCULATIONS

After subtraction of the blank, the antibody-bound fraction (B_i) was expressed either as a percentage of the total radioactivity (B_i - B_b/T $\times$ 100) or as a percentage of the maximum binding (B_i - B_b/B₀ $\times$ 100). The best cubic curve was computed for the standards using the method of least squares, and the amount of T₄ in the test samples was interpolated automatically using a suitable computer program and a 9100b calculator (Hewlett-Packard).

Manual methods of calculation, although more time consuming, could be employed which gave results identical with those obtained by computerized techniques.

INVESTIGATION OF EFFECT OF BARBITAL BUFFER ON PRE-ALBUMIN BINDING

A standard curve, 10 samples, and the three quality control sera were measured in duplicate (a) in barbitol buffer, pH 8.6 and (b) in phosphate buffer, pH 7.4.

QUALITY CONTROL SCHEME

Control sera: A pool of normal serum (the normal quality control, NQC) was prepared, and its T₄ content was determined repeatedly and found to be approximately 100 nmol/l. An aliquot of this serum was diluted 1:2 with T₄ free serum to give the low quality control serum (LQC) containing approximately 50 nmol/l, and standard T₄ was added to another aliquot to give the high quality control serum (HQC) containing approximately 200 nmol/l. These three pools were aliquoted and stored at -20°C.

To assess intralaboratory variation the 'within-batch' coefficient of variation was assessed by 10 simultaneous analyses of each of the quality control sera, and the 'between-batch' by analysis of the NQC in duplicate in 61 consecutive assays.

To assess interlaboratory variation the three quality control sera were sent by post to six different laboratories where the T₄ content was determined using the same reagents and a protocol identical with that described in this paper.

COLLECTION OF BLOOD SAMPLES

(a) Blood samples were collected from 109 healthy laboratory personnel, aged 16 to 45 years, comprising 61 men and 48 women of whom 18 were taking oral contraceptives. An additional series of samples from 86 clinically euthyroid subjects was kindly supplied by Professor R. Hall. These subjects, comprising 39 men and 46 women

aged 18 to 87 years, all showed a normal thyrotrophin (TSH) response to the thyrotrophin releasing hormone (TRH) test (Ormston *et al*, 1971), indicating that they were also 'biochemically euthyroid'.

(b) To assess any circadian changes, six men and five women, aged 21 to 34 years, had repeated venepunctures at 2-hourly intervals between 8 am and 8 pm, and also at midnight and at 4 am.

(c) To compare serum and plasma, blood samples were collected from 10 clinically euthyroid subjects, half of each sample being collected into a plain glass tube and allowed to clot, while the remainder was collected into a lithium heparinized tube (containing approximately 10 U heparin/ml).

All samples, unless otherwise stated, were serum stored at -20°C before assay.

Results

A typical standard curve is shown in figure 1. Blank values were approximately 10%. The 'within-batch' coefficient of variation for the low, normal, and high quality control sera were 5.6, 2.5, and 6.2% respectively, and the 'between-batch' coefficient of variation for the normal quality control serum was 5.5%. Table I shows the values for the three quality control sera when measured by six different laboratories using this method and these were considered satisfactory.

Doubling dilutions of six serum samples were prepared using T₄ free serum and their T₄ content was measured. Figure 1 shows parallelism with the standard curve.

Fig 1 Parallel inhibition of the binding of ¹²⁵I-T₄ exhibited by serial dilutions of serum samples (-----) compared with standard solutions (——).

Hospital	T ₄ in nmol/l			Between-assay ¹ Coefficient of Variation
	LQC	NQC	HQC	
SBH	50	99	197	5.5
1	48	92	180	5.5
2	71	114	219	7.9
3	48	102	199	5.4
4	43	103	194	—
5	46	90	170	4.6
6	39	90	180	2.9
Mean	49	100	191	
SD	10.2	8.7	16.9	
CV	20.8	8.7	8.7	

Table I T₄ values of three quality control sera, as measured by seven different laboratories

¹These figures were obtained for serum with T₄ values which were within the normal range. The number of determinations varied between 6 and 61.
SBH = St. Bartholomew's Hospital

Figure 2 shows standard curves obtained with barbital buffer, pH 8.6 and phosphate buffer, pH 7.4. The difference in binding was probably due to pH effects on the antigen:antibody reaction as demonstrated in a triiodothyronine RIA (Hüfner and Hesch 1976) where higher binding was obtained at pH 7.4 than at 8.6. The total T₄ values for the 10 serum samples and three quality control sera were similar when measured in both buffer systems (table II), indicating that in the presence of a high avidity antiserum to T₄ the effect of pre-albumin binding on results is insignificant.

Results for 177 normal euthyroid subjects and 18 females taking oral contraceptives are shown in figure 3. Excluding the latter, there was no sex difference, the mean serum total T₄ for women being 107 ± 21 and for men 103 ± 17 nmol/l. Total T₄ levels did not decrease with age, thus the mean

Fig 2 Comparison of standard curves in phosphate buffer (●) and barbital buffer (○).

Sample	Buffer	
	Phosphate, pH 7.4	Barbital, pH 8.6
	T ₄ in nmol/l	
1	45	42
2	55	50
3	72	74
4	76	74
5	86	99
6	105	112
7	107	104
8	118	119
9	150	156
10	153	157
LQC	48	50
NQC	102	103
HQC	185	199
Mean	100.1	103.0
SD	±43.1	±46.8

Table II Serum T₄ levels measured in phosphate and barbital buffer

Fig 3 Distribution of T₄ levels (A) in 18 female subjects taking oral contraceptives and (B) in 177 normal euthyroid subjects.

values for 140 subjects aged 16 to 59 years was 105 ± 19 and for 37 aged 60 to 87 was 103 ± 16 nmol/l. The mean for the total normal population was 105 ± 19 . Although the mean serum total T₄ for the subjects taking oral contraceptives was significantly

higher (130 ± 30 nmol/l; $P < 0.001$), only three had levels which were outside the normal range of 57 to 155 nmol/l.

The results of the circadian rhythm study are shown in table III. Although the coefficients of variation for individuals were slightly higher than that of the method, no definite pattern was observed.

Similar results were obtained for serum and lithium heparinized plasma, the mean total T₄ levels for the 10 subjects studied being 94.7 ± 13.9 and 94.2 ± 14.4 nmol/l respectively.

Discussion

Thyroid disorders are among the commonest endocrine abnormalities, and, therefore, most chemical pathology laboratories receive large numbers of requests which necessitate a simple screening procedure in order to cope with the ever increasing demand.

Until recently, total T₄ levels were determined either indirectly as PBI or more directly by protein-binding methods. The results obtained, however, may be affected by non-specific interference. In an investigation of thyroid disease in geriatric subjects, Jefferys (1972) showed that at least one patient in 10 had a spuriously raised PBI attributable to iodine contamination while, in a study of the stability of thyroid hormones (Nye *et al*, 1975), it was shown that when plasma and serum samples are stored at room temperature their apparent T₄ content increases significantly when measured by protein-binding methods but remains stable for at least two weeks when measured by radioimmunoassay. Rootwelt (1975) has demonstrated that this apparent increase in total T₄ is due to non-specific interference by free fatty acids which are released slowly on storage. This instability problem is obviously important when samples are sent by post. Thus radioimmunoassay is the only specific method which is available for the measurement of total T₄.

Radioimmunoassays for T₄ are also simpler and

Subject	Age	Sex	Time throughout Day									Mean	SD	CV%
			08.00	10.00	12.00	14.00	16.00	18.00	20.00	00.00	04.00			
RW	34	M	—	112	84	105	95	92	95	98	95	97	8.4	8.7
ADH	22	M	130	—	123	105	109	118	116	124	125	119	8.5	7.1
JK	30	M	—	93	103	104	106	101	98	97	93	99	4.9	5.0
VC	25	F	155	165	164	127	147	159	157	134	131	148	14.6	9.9
JD	23	F	131	150	145	140	129	138	97	97	—	128	20.5	16.0
CB	26	F	84	95	104	92	84	89	88	100	90	92	6.8	7.4
JG	26	F	111	112	116	90	97	85	94	84	90	98	12.2	12.5
EN	21	M	—	102	109	109	106	101	121	110	115	109	6.6	6.0
SD	21	F	100	114	111	118	107	119	109	108	89	108	9.3	8.6
AET	21	M	103	—	61	80	69	68	77	72	92	77	14.3	18.6
HL	25	M	69	84	—	73	77	99	68	62	58	74	13	17.7

Table III T₄ levels measured by RIA in the circadian rhythm study

quicker to perform than protein-binding techniques which require extraction of T₄ from endogenous binding proteins before assay. In addition, they are much less expensive than commercially available T₄ kits, based on protein-binding methods. Preparation and distribution costs of anti-T₄ serum are negligible; the sheep employed in this study was bought and maintained for approximately £15 per month, and the antiserum was despatched to other laboratories by post in an unfrozen state. With regular immunization and bleeding procedures one animal will produce enough antiserum to supply an entire region for several years. Thus, considering reagents alone, for a commonly employed binding kit, the cost per tube is at least 100 times greater than for the RIA described here (table IV).

The normal values obtained in this study compare closely with those of other workers employing protein binding or radioimmunoassays (table V). It has been in routine use in this laboratory for a year and is currently employed in at least 10 other hospitals, of which six took part in a joint quality control scheme. Both 'within' and 'between' laboratory coefficients of variation were less than 9%

RIA		Kit	
Reagent	Cost/100 tubes £	Reagent	Cost/100 tubes £
Buffer	0.006	Kit reagents	63.6
¹²⁵ I-T ₄	0.525	Ethanol	0.1
ANS	0.001		
PEG 6000	0.023		
Total reagent cost/100 tubes	£0.555	Total reagent cost/100 tubes	£63.7

Table IV Comparison between the reagent costs of the T₄ RIA and a commonly used protein-binding kit

Protein-binding Assay			Radioimmunoassay		
Author	Mean ± SD	Range	Author	Mean ± SD	Range
Murphy and Pattee (1964)	130 ± 26	—	Chopra (1972)	107 ± 31	—
Ekins <i>et al</i> (1969)	—	58–148	Mitsuma <i>et al</i> (1972)	95 ± 25	50–126
Braverman <i>et al</i> (1971)	95 ± 19	—	Beckers <i>et al</i> (1973)	122 ± 25	—
Alexander and Jennings (1974)	—	58–154	Ratcliffe <i>et al</i> (1974b)	99 ± 21	55–144
			Meinhold and Wenzel (1974)	103 ± 21	71–151
			Black <i>et al</i> (1974)	120	75–172
			Marsden <i>et al</i> (1975)	81 ± 14	—
			Present study	103 ± 21	57–155

Table V Normal T₄ levels as reported in the literature (converted to nmol/l)

except for the low quality control serum in which discrepant results of 71 nmol/l and 39 nmol/l obtained from two different hospitals increased the 'between' laboratory coefficient of variation to 20.8%. A regular exchange of standard material and control sera is in progress and it is hoped that 'between' laboratory variation will improve with time.

The preparation of reagents is simple, although the extraction of T₄ from serum is a time-consuming process. Fortunately, large batches can be prepared at one time and are stable for at least six months when stored at -20°C. Marsden *et al* (1975) described a T₄ RIA in which T₄ free serum was not required provided that serum samples were diluted between 1:10 and 1:100 with buffer. This predilution step is, however, also time-consuming, especially when large numbers of samples are being processed.

The assay may be operated either manually in the small laboratory or adapted for use with semi-automated equipment (such as the Compupet and Micromedic pipetting systems) when larger batches of samples are assayed. Both lithium heparinized plasma and serum samples are suitable. No circadian rhythm was observed, and blood samples may therefore be collected at any time.

In most cases of suspected thyroid dysfunction a diagnosis can be substantiated by the estimation of total serum T₄ levels together with a simple thyroid hormone uptake test, such as the Thyopac 3 kit (Radiochemical Centre), which gives a measure of available binding sites on circulating TBG. The Thyopac 3 assay and the T₄ radioimmunoassay reported here can easily be carried out within a working day and constitute a satisfactory screening technique. In most cases no further tests need be done.

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