Technical method

A rapid and simple method for simultaneous measurement of serum free thyroxine and triiodothyronine fractions

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The physiological importance of non protein-bound thyroid hormone in serum has been recognized for many years (Recant and Riggs, 1952; Robbins and Rall, 1957). Because of high-affinity binding by serum proteins, the fraction of free or unbound hormone is relatively small and its measurement presents considerable difficulties. An index of serum free thyroxine may be calculated from serum total thyroxine and the concentration of unoccupied binding sites on serum proteins (MacLagan and Howorth, 1969; Wellby *et al.*, 1973). More direct estimates of the free hormone fraction may be obtained by addition of a radioactive isotope to serum and subsequent separation of free from protein-bound hormone by gel filtration (Lee *et al.*, 1964; Sakurada *et al.*, 1969), ultrafiltration (Thorson *et al.*, 1972) or equilibrium dialysis (Oppenheimer *et al.*, 1963; Ingbar *et al.*, 1965; Sterling and Brenner, 1966). Dialysis of serum against a buffer solution across a membrane permeable to free but not to protein-bound hormone is the most widely used method of separation despite its several disadvantages. Prolonged incubation, necessary to achieve equilibrium, ensures that the procedure is usually carried over to the next working day. A high degree of technical skill is needed to avoid radioactive contamination of dialysate. Most importantly, a further step is necessary to separate radioactivity due to hormonal iodine from that due to contaminating inorganic iodine which is also freely diffusible and constitutes the greater part of radioactivity in dialysate.

The technique described here is a considerable modification of an earlier method (Lee *et al.*, 1964) in which the properties of Sephadex gel are used to separate free from protein-bound hormone and contaminating inorganic iodine. By the simultaneous use of two iodine radioisotopes a simple, rapid,

inexpensive, and reliable measurement of serum free thyroxine and triiodothyronine fractions may be obtained.

Material and methods

RADIOACTIVE HORMONES

Thyroxine labelled with ^{131}I (specific radioactivity approximately 50 mCi/mg) was obtained from the Radiochemical Centre, Amersham, Bucks. Immediately after receipt it was diluted to a concentration of approximately 1000 nmol/l with 2% lyophilized bovine albumin solution and used for a maximum of two weeks. Shipments of radioactive hormone were tested chromatographically and rejected if less than 95% pure.

Triiodothyronine labelled with ^{125}I (specific radioactivity approximately 500 mCi/mg) was obtained from Abbott Laboratories, Chicago, USA. Solutions containing approximately 15 nmol/l were made up in 2% lyophilized bovine albumin. Stock radioactive hormone solution was checked chromatographically from time to time and was always found to be at least 97% pure.

HARTMANN'S SOLUTION (Compound Sodium Lactate BP)

Obtained from Baxter Division, Travenol Laboratories Ltd. Before use pH was adjusted to 7.4 by addition of dilute sodium hydroxide.

SODIUM CHLORIDE SOLUTION

0.5 M; pH 7.0.

SEPHADEX GEL

Sephadex G25 (coarse) was purchased from Pharmacia Fine Chemicals. The dry powder was allowed to swell overnight in EDTA solution (3.7×10^{-2} M; pH 7.0) in the ratio 100 mg of powder to 5 ml of EDTA solution.

COLUMNS

Each column consists of the barrel of a plastic disposable 1 ml insulin syringe (Steriseal Ltd, Redditch, Worcester), the outlet of which is sealed with a punched-out sintered disc (fig 1). Columns are filled exactly to the 0.5 ml mark with Sephadex slurry and then washed through with 2 ml of EDTA solution to ensure packing. The syringe tip is blocked with a removable plastic cap. Columns are stored at room temperature, excess EDTA solution lying above the gel.

Fig 1 Column employed in the assay procedure.

PRINCIPLE

Sephadex has two properties which allow of its use in the separation of protein-bound from free hormone: (a) it acts as a molecular sieve so that small molecules are retained on the gel while large molecules are washed off; (b) it binds phenolic compounds. When serum is run through a Sephadex column, which is subsequently washed, protein-bound hormone is eluted whereas the relatively small molecular weight unbound phenolic thyroxine and triiodothyronine are retained. Previous equilibration of serum with radioactive hormone allows the relative proportions of protein-bound and free hormone to be calculated from radioactivity present in effluent and remaining on the column.

Procedure

(1) 0.2 ml of serum is pipetted into a small disposable plastic tube. 0.01 ml each of ^{131}I thyroxine solution and ^{125}I triiodothyronine solution is added. This produces increments in stable hormone concentrations of approximately 50 nmol/l and 0.75 nmol/l respectively. Tubes are thoroughly mixed and allowed to incubate at room temperature for at least one hour.

(2) Labelled serum is diluted 1 in 10 by the addition of 1.8 ml of Hartmann's solution. Tubes are left for at least 30 minutes.

(3) Sephadex columns are allowed to run dry by removal of the plastic cap. 0.5 ml of dilute labelled serum is slowly pipetted onto each column at a rate which permits its immediate entry into the resin bed and avoids contact with the syringe barrel. The effluent is collected into a 10 cm disposable plastic tube.

(4) Each column is washed with 2.5 ml of sodium chloride solution and the effluent is collected into the same tube, thus giving a final effluent volume of 3 ml. This step should be completed within three minutes of the previous step. It is possible to elute up to 26 columns in this time by washing in rotation with 5×0.5 ml aliquots using a Lumex automatic dispenser.

(5) The effluent volume is made up to 5 ml with sodium chloride solution and the tubes are thoroughly mixed.

(6) 0.5 ml aliquots of effluent are placed in small disposable plastic tubes which have dimensions similar to those of the columns when the barrel outlets of the latter are broken off. Both tubes and columns are counted to an accuracy of at least 95% in an automatic gamma-counter. This is achieved by counting for 20 minutes in the case of columns and two minutes in the case of tubes.

Calculation

The aliquot of effluent counted contains 1% of the protein-bound activity present in original undiluted serum. Since the absolute free hormone concentration is largely unaffected by serum dilution over a wide range (Oppenheimer and Surks, 1964), activity remaining on the column, which is derived from serum free hormone, is independent of the dilution factor. Thus:

$$\text{Free hormone fraction} = \frac{\text{Activity on column}}{\text{Activity in effluent} \times 100}$$

$$\% \text{ Free hormone} = \frac{\text{Activity on column}}{\text{Activity in effluent}}$$

Specimens of blood were obtained from the following: (a) 20 euthyroid normal adults; (b) 12 patients with clinical and biochemical evidence of thyrotoxicosis; (c) seven patients with clinical and biochemical evidence of hypothyroidism; (d) 10 patients in the last trimester of pregnancy; and (e) 10 apyrexial hospital inpatients suffering from a variety of moderately severe subacute or chronic non-thyroidal illness.

No individual was receiving drug therapy known to interfere with thyroid function tests.

Serum was stored at -20°C and thawed immediately before use. Analyses were carried out in duplicate.

Serum total thyroxine and triiodothyronine were respectively measured by competitive protein binding and radioimmunoassay techniques, as described by Black *et al* (1975).

Results

METHODOLOGICAL VALIDATIONS

The elution patterns for inorganic iodine, protein, and thyroid hormones in protein-free solution were determined as described below. Six columns were employed in each set of experiments.

(a) Inorganic iodine: 0.5 ml of a solution of ^{131}I sodium iodide was applied to the column which was then washed with 2.5 ml of sodium chloride solution. Mean radioactivity retained on the column was 0.5% of that applied (range 0.0–0.8%). Since no more than 5% of radioactivity in test serum is due to inorganic iodine, the error resulting from retention of this contaminant on the column is negligible.

(b) Protein: 0.5 ml of a solution of ^{125}I iodinated albumin was applied to the column. After elution with 2.5 ml of sodium chloride less than 0.1% of applied radioactivity was retained on the column (mean 0.04%; range 0.01–0.08%). If protein-bound radioactive hormone has a similar elution pattern, then up to 0.08% of protein-bound radioactive hormone might remain on the column and this would constitute a serious error. However, use of serum diluted 1 in 10 results in the retention of no more than 0.008% of protein-bound radioactivity present in the original undiluted serum. Washing with excess sodium chloride solution did not achieve more complete elution.

(c) Thyroid hormones: in separate experiments, 0.5 ml aliquots of ^{125}I triiodothyronine and ^{131}I thyroxine in protein-free Hartmann's solution were applied to the column which was then washed with 2.5 ml of sodium chloride solution. Mean radioactivity retained on the column was 89% of that applied in the case of thyroxine (range 81–90%) and 85% in the case of triiodothyronine (range 80–87%). Eluted radioactivity consisted of inorganic iodine (approximately 4% of original added radioactivity) and hormone (approximately 10% of original radioactivity). Washing with larger volumes of sodium chloride solution resulted in a further loss of radioactivity from the column.

Thus the elution step results in quantitative removal of inorganic iodine, retention of an insignificant amount of protein-bound radioactivity, and elution

of approximately 10% of unbound thyroid hormone.

Effects of serum enrichment with stable hormone

No change in serum free hormone fraction was apparent when sufficient radioactive material was added to increase the serum total thyroxine concentration over the range 20–100 nmol/litre and the serum total triiodothyronine over the range 0.5–2.0 nmol/litre. It may be concluded that the increments in serum total hormone concentrations after the labelling procedure (respectively 50 nmol/l for thyroxine and 0.75 nmol/l for triiodothyronine) do not appreciably alter the free hormone fraction.

Effects of serum dilution

Values for the free hormone fraction in undiluted serum and at various dilutions up to 1 in 10 are shown in figure 2. Dilution results in calculated mean values which are 10–15% less than those obtained with undiluted serum. This effect is apparent at a 1 in 2 dilution, and further dilution does not appreciably alter the free hormone fraction.

Fig 2 Effect of progressive serum dilution on the measured free fractions of thyroxine and triiodothyronine. Values are expressed as a percentage of that found in undiluted serum. Mean values ± 1 SD are shown.

Use of radioactive hormones singly and together

Values for free hormone fractions obtained individually by the use of one radioactive hormone only did not differ from those obtained by the simultaneous use of both radioactive hormones.

Reproducibility

This was assessed by measuring the free hormone fraction in 10 aliquots of pooled normal serum during a single assay procedure and by incorporating an aliquot from a second serum pool in each of 10 consecutive assay runs. Inter- and intra-assay coefficients of variation were respectively 7.3% and 9.1% in the case of thyroxine and 5.3% and 7.0% in the case of triiodothyronine.

The ratio between the volumes of dilute serum and gel bed is arbitrary but closely approximates that described by Lee *et al* (1964). Increases in apparent free hormone fraction are seen when this ratio is smaller. Thus application of 0.1 ml of dilute serum to the standard column results in a 10-fold increase in calculated free thyroxine fraction. A delay of more than five minutes in instituting and completing the saline elution step leads to large and unpredictable increases in the serum free thyroxine fraction.

Columns must be prepared with great care. A high degree of pipetting accuracy is essential. Radioactive hormone of high purity should be employed.

With these provisos, the method is simple and good reproducibility may be achieved.

PATIENT STUDIES

Values for serum total, percentage free fraction and serum free concentration of thyroxine and triiodothyronine are shown in the table. The latter variables best distinguished euthyroid subjects from those with thyroid malfunction. Whereas values for the serum free thyroxine concentration in pregnant patients showed some overlap with those in the hypothyroid group, the serum triiodothyronine concentration clearly discriminated between euthyroid subjects and those with thyroid disease (fig 3).

Discussion

This method differs from that of Lee *et al* (1964) in several important respects: (1) Columns are made from easily available, inexpensive, disposable syringes of uniform dimensions which need no special preparation, whereas in the original method columns were individually made up by drawing soft glass and then subjecting it sequentially to acid washing, water rinsing, immersion in Desicote, and finally, acetone washing. (2) The system has been miniaturized so that smaller volumes of serum are needed but the ratio of serum/gel bed volume is maintained. (3) Dilute rather than neat serum is employed. In the method of Lee and his co-workers 1 ml of serum is needed per single assay. In practice it is preferable to assay in duplicate, and thus 5 ml of blood is needed for each assay. Duplicate analyses by the present method may be carried out on 0.2 ml of serum. In order to achieve sufficient radioactivity in the non protein-bound fraction, it is necessary to add a relatively large amount of tracer to undiluted serum. Automatic counting is greatly facilitated if serum is diluted following its equilibration with radioactive hormone. Since small aliquots only of undiluted labelled serum may be counted efficiently pipetting errors are likely to be greater. (4) The elution step for free thyroxine has been eliminated thus appreciably shortening the assay procedure. (5) The use of two radioactive iodine isotopes allow simultaneous measurement of free thyroxine and triiodothyronine fractions.

The improved reproducibility of the present method over that of Lee *et al* (1964) is probably attributable to the use of more uniform columns and dilution of serum before column chromatography.

There is good evidence that the uptake of free

Group (No.)	Serum thyroxine			Serum triiodothyronine		
	Total (n mol/l)	% Free fraction	Free conc. (p mol/l)	Total (n mol/l)	% Free fraction	Free conc. (p mol/l)
Normal (20)	121 ± 18 (94 - 157)	0.052 ± 0.005 (0.044 - 0.062)	63 ± 10 (51 - 86)	2.3 ± 0.5 (1.7 - 3.3)	0.40 ± 0.06 (0.29 - 0.50)	9.1 ± 2.2 (5.4 - 12.1)
Thyrotoxic (12)	*279 ± 73 (167 - 391)	*0.124 ± 0.036 (0.058 - 0.178)	*353 ± 154 (136 - 600)	*8.2 ± 3.6 (4.5 - 14.3)	*0.52 ± 0.10 (0.35 - 0.73)	*41.8 ± 18.3 (24.7 - 84.2)
Hypothyroid (7)	*51 ± 22 (19 - 79)	*0.031 ± 0.009 (0.015 - 0.039)	*18 ± 10 (3 - 30)	*1.1 ± 0.3 (0.8 - 1.7)	*0.20 ± 0.02 (0.17 - 0.23)	*2.3 ± 0.8 (1.4 - 4.0)
Pregnant (10)	*165 ± 24 (139 - 206)	*0.022 ± 0.005 (0.013 - 0.030)	*36 ± 9 (26 - 50)	*3.6 ± 0.7 (2.8 - 5.0)	*0.20 ± 0.05 (0.12 - 0.30)	7.2 ± 2.0 (5.2 - 11.1)
Euthyroid sick (10)	126 ± 17 (106 - 162)	0.063 ± 0.015 (0.044 - 0.099)	80 ± 27 (53 - 142)	2.3 ± 0.7 (1.3 - 3.5)	0.36 ± 0.07 (0.29 - 0.50)	8.4 ± 3.2 (4.9 - 14.7)

Table Values for serum, total percentage free fraction, and absolute serum free concentration of thyroxine and triiodothyronine

Means ± 1 SD (range in brackets) are presented. The significance of differences from normal in other clinical groups was assessed by means of Wilcoxon Rank testing.

*P < 0.001

Fig 3 Serum free thyroxine and triiodothyronine values in normal, thyrotoxic, hypothyroid, pregnant, and euthyroid sick patients.

hormone by Sephadex is rapid (Cavalieri *et al*, 1969) and that no significant shift of hormone from serum binding proteins to gel takes place (Irvine, 1974).

Despite theoretical considerations and experimental evidence which suggest that the serum free thyroxine concentration is unaltered by dilution over a wide range (Oppenheimer and Surks, 1964), most workers employing dilute serum (Strickler *et al*, 1974; Wilson *et al*, 1974) report values for the free hormone fraction which are considerably lower than those recorded when undiluted serum is used (Ingbar *et al*, 1965; Sterling and Brenner, 1966). This dilution effect may be accounted for by the use of non-physiological buffers. It has been shown that the free thyroxine fraction is proportioned to the chloride ion concentration over a wide range (Spaulding and Gregerman, 1972; Irvine, 1974). The latter is greatly decreased when serum is diluted with the commonly employed phosphate buffer. The serum free hormone fraction is also proportional to the albumin concentration (Lee and Pileggi, 1971). In the method described here, the chloride ion concentration is kept in the physiological range by the use of Hartmann's solution as diluent. It is possible that the small reductions in hormone free fraction apparent at a low dilution are due to reduced albumin concentration.

Previous experiments with unlabelled material indicated that, despite its weak buffering capacity, no significant pH change occurred in dilute serum in

the context of the assay procedure. Difficulty in maintaining the pH within narrow limits for long periods is one of the problems associated with equilibrium dialysis. The free hormone fraction is inversely related to the pH (Lee and Pileggi, 1971; Thorson *et al*, 1972).

Values for the serum free fraction and free thyroxine concentration in normal, thyrotoxic, and hypothyroid patients similar to those recorded in the present study have been reported by other workers employing equilibrium dialysis (Ingbar *et al*, 1965; Sterling and Brenner, 1966; Liewendahl and Lamberg, 1969), ultrafiltration (Thorson *et al*, 1972), and Sephadex gel filtration (Sakurada *et al*, 1969). Comparable patterns of serum total, free fraction and the resultant free thyroxine concentration have been reported in pregnancy (Ingbar *et al*, 1965; Sterling and Brenner, 1966; Thorson *et al*, 1972) and non-thyroidal illness (Ingbar *et al*, 1965; Sterling and Brenner, 1966; Arango *et al*, 1968).

Relatively few studies on serum free triiodothyronine levels in various clinical states have been carried out. Accurate values for absolute free hormone concentration have been obtainable only since the advent of reliable radioimmunoassay methods for serum total triiodothyronine measurements. The present study indicates that the serum free triiodothyronine concentration is approximately 10% of the free thyroxine concentration at all levels of thyroid function. Values for the serum

free fraction in normal, hypothyroid, and thyrotoxic subjects which closely approximate those in the present study have been reported by earlier groups using equilibrium dialysis (Nauman *et al*, 1967) or gel filtration (Sakurada *et al*, 1969). It is interesting to note that values for the serum free triiodothyronine concentration in normal subjects obtained by direct radioimmunoassay of unlabelled serum dialysates (Ellis and Ekins, 1973) are virtually identical with those reported here.

Decreases in the free fraction of triiodothyronine in pregnant patients similar in magnitude to those reported in this study were found by Sakurada *et al* (1969). Less striking reductions in the free fraction with normal serum free hormone concentration were reported by Abuid *et al* (1974).

No significant difference from normal in either the free fraction or free hormone concentration was apparent in euthyroid sick patients. This may reflect the severity of illness present. A marked depression in serum triiodothyronine and an increase in serum free fraction with resulting decrease of absolute free triiodothyronine concentration has been reported in patients with very severe illness (Carter *et al*, 1974).

Extended studies, necessary to define more exactly the normal range of free thyroxine and triiodothyronine concentrations, are at present being carried out.

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