Measuring Free Thyroxin by Using Magnetic Antibody-Containing Microcapsules

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We describe a novel magnetic-separation microencapsulated antibody displacement assay for free thyroxin. The method is rapid (results available in just over an hour), with a detection limit of 3 pmol/L, and an interassay precision of <10% over the range 8–75 pmol/L. Results are not influenced by changes in the concentration of thyroxin-binding globulin or albumin, nor by the presence of circulating anti-thyroxin antibodies or physiological concentrations of nonesterified (free) fatty acids. A reference interval of 8.4–18.4 pmol/L was established for a large euthyroid adult population. Patients with various nonthyroidal illnesses exhibited a similar reference interval (6.2–17.4 pmol/L). Results from pregnant women, at all stages of gestation, were within the nonpregnant euthyroid reference interval. Complete discrimination was obtained between the euthyroid reference interval and values from patients with untreated thyrotoxicosis (24–75 pmol/L). Excellent, though incomplete, discrimination was obtained for patients with untreated hypothyroidism (<3.0–9.0 pmol/L). Results for a group of patients defined as having compensated euthyroidism (5.0–15 pmol/L) showed considerable overlap with the euthyroid reference interval. Patients taking thyroxin, who were clinically euthyroid and whose serum concentration of thyrotrpin was within its euthyroid reference interval, had free thyroxin concentrations in the range 13.4–24.2 pmol/L. We discuss the validity of this assay in clinical practice.

Additional Keyphrases: thyroid status · reference interval

It is generally, but not universally (1), agreed that measurement of free thyroxin (FT₄) in serum can help to reduce diagnostic errors during thyroid-function testing (2). Unfortunately, the reference method for determining FT₄ (3–5), equilibrium dialysis, is too cumbersome for routine use in most clinical laboratories, so various other methods have been developed, all of which aim at simplifying the procedure without decreasing clinical discrimination or assay validity.

The use of a T₄-analog label, pioneered by Midgley and Wilkins (6), has proved surprisingly popular, even though there are well-documented interferences (7) and the validity of the method has been seriously questioned (8). Various other approaches, e.g., sequential two-step, labeled-antibody or labeled-antigen assays, have been described (9) and are available commercially. The clinical performance for most of these methods has been described in some detail (2, 10, 11).

Ten years ago, the potential of microencapsulated anti-

body for measuring of FT₄ was first outlined (12, 13). Reagents based on this principle and requiring centrifugation to separate bound and free fractions were commercially available during the early part of this decade. The assay relied on physical retention of antibodies within semipermeable microcapsules so that dialysis and immunoassay could occur in a one-step procedure. Microencapsulated antibody assays have also been developed for measuring other analytes, e.g., digoxin (14), cortisol (15), and testosterone (16) ("Liquisol", Damon Diagnostics, Boston, MA).

More recently we have developed a procedure for antibody encapsulation, based on interfacial polymerization (17), that has been successfully used to prepare the antibody reagent for an extremely robust, large-scale screening assay for the measurement of 17-hydroxyprogesterone in neonates (18). The technical simplicity of the procedure is greatly advantageous and has allowed a large number of blood-spot samples to be screened over a two-year period. The microcapsule membrane has a pore size that allows entry of compounds of molecular mass <4000 Da (19), i.e., large enough to allow entry of thyroxin but not of thyroid hormone-binding proteins. Consequently, we determined to assess the use of magnetic antibody-containing microcapsules in a simple direct assay for FT₄.

Materials and Methods

Materials

Apparatus. Gamma radiation was measured with an NE-1600 Counter (Nuclear Enterprises, Edinburgh, U.K.). To agitate solutions during the emulsification stage of the microencapsulation procedure, we used an Ultra-Turrax disperser operated with an 18 KG solvent-resistant shaft supplied by Semat (U.K.) Ltd., St. Albans, U.K. For centrifugation we used a "Coolspin" centrifuge (M.S.E Scientific Instruments, Crawley, U.K.). For magnetic separation we used flat-bed magnetic racks, each able to hold 50 × 75 mm glass tubes as supplied from Immunodiagnostic Systems Ltd., Tyne and Wear, U.K. For repeat dispensing of assay reagents we used an adjustable BCL 8000 pipette (Boehringer Mannheim, Lewes, East Sussex, U.K.).

Reagents and existing methods. All common chemicals and solvents were of ANALAR grade and supplied from either B.D.H. Chemicals Ltd., Dorset, U.K., or Sigma Chemical Co., Dorset, U.K. Carrier-free [¹²⁵I]-l-thyroxin, specific activity 4.4 × 10⁶ Ci/mol, was supplied by New England Nuclear, Boston, MA 02118. Phosphate buffer (0.1 mol/L, pH 7.4) containing 9 g of NaCl and 1 g of sodium azide per liter was used throughout.

We measured serum albumin by the bromcresol green dye-binding method, as automated on the BM/Hitachi 704 analyzer (Boehringer Mannheim; "normal" reference range 35–54 g/L). Thyroxin-binding globulin (TBG) was measured with the Corning "Immuno Phase" radioimmunoassay (Corning Medical, Medfield, MA 02052) (reference range 12–30 mg/L). Total thyroxin (TT₄) and triiodothyronine (TT₃) were measured with in-house radioimmunoassays (reference ranges 55–144 nmol/L and 0.9–2.8 nmol/L, re-

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2 Nonstandard abbreviations: FT₄, free thyroxin; TT₄, total thyroxin; TT₃, total triiodothyronine; TBG, thyroxin-binding globulin; and TSH, thyrotrpin.
3 Received September 29, 1989; accepted January 15, 1990.
spectively). Thyrotropin (TSH) was measured with an in-house immunoradiometric assay (reference range 0.3–5.0 milli-int. units/L); samples with values <0.4 milli-int. units/L were assayed with the “Delfia” TSH assay (Pharmacia Ltd., Milton Keynes, U.K.), which has a detection limit of 0.05 milli-int. units/L. Anti-thyroxin antibody for encapsulation was obtained from Dr. T. Merrit, Rast Allergy Unit, Beneden Chest Hospital, Kent, U.K. For analog-based FT\textsubscript{4} measurements we used the Amerlex-M FT\textsubscript{4} procedure as supplied by Amersham International plc, Amersham, Bucks, U.K.

We used as a separating aid magnetic cellulose, prepared by a method similar to that described by Dawes and Gardiner (20), at a concentration of 1 g/L in phosphate buffer containing 50 g of Polyethylene Glycol 6000 per liter.

Standards. FT\textsubscript{4} standards were prepared by dissolving increasing amounts of thyroxin in analyte-free serum (charcoal-stripped human serum, as supplied by Scantibodies Laboratories Inc., Santee, CA 92071). These standards were calibrated against reference preparations validated by the equilibrium dialysis method of Ekins (21).

Precision profiles. Intra-assay precision profiles were calculated, as described by Ekins and Edwards (22), with an Apple II microcomputer, with the use of the WHO-Data-Processing Program developed at the Middlesex Hospital Medical School, London, U.K.

Methods

Antibody encapsulation: Microcapsules containing antibody raised against thyroxin were prepared as described previously (17) except for the incorporation of magnetic dextran instead of bovine serum albumin. Magnetic dextran was prepared as described by Molday and Mackenzie (23). Microencapsulated antibody (about 50 000 capsules/mL) was pre-incubated (37°C, 1 h) with an appropriate quantity of carrier-free (\textsuperscript{125}I)\textsubscript{L}-thyroxin and washed with phosphate buffer (pH 7.4) to yield capsules containing 0.1 μCi of radiolabel per milliliter.

FT\textsubscript{4} assay procedure: Add 100 μL of standard, quality-control serum, or patient's serum to 500 μL of radioactivity-labeled microencapsulated antibody and incubate for 45 min at 37°C. Mix with 1 mL of magnetic separating agent and allow the contents of the tubes to sediment on magnetic trays for 15 min. Remove the supernatant liquid by inversion and let the tubes drain for 5 min. Quantify for 1 min the radioactivity remaining in each tube.

Although magnetic microcapsules will sediment unaided when the tubes are placed on the magnetic rack, the addition of magnetic cellulose at the end of the incubation speeds up this sedimentation and produces a more stable pellet in which magnetic antibody-containing microcapsules are trapped. The proportion of radiolabeled thyroxin displaced from the microencapsulated antibody during incubation with serum increases with time (Figure 1). The assay incubation time of 45 min is based on yielding adequate sensitivity with an acceptable degree of displacement over the concentration range of the standard curve.

Results

Assay Characteristics

Imprecision: A typical standard curve and intra-assay precision profile is shown in Figure 2. Interpolated from the precision profile, the range of the standard curve was 3–75 pmol/L, the detection limit being defined as the point at which the CV was <22% (24). Intra- and interassay imprecision values obtained with quality-control sera are documented in Table 1. The reported interassay CVs accord with those of most other FT\textsubscript{4} methods reported and are considered clinically acceptable.

Dilution experiments: Figure 3 shows results for serum FT\textsubscript{4} concentrations, measured before and after dilution with assay buffer, in serum pools obtained from five hypothyroid, euthyroid, or thyrotoxic patients. During the course of a standard assay, the sample dilution is sixfold, with reasonable linearity of response (parallelism with the

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|}
\hline
& QC1 & QC2 & QC3 & QC4 \\
\hline
\textbf{Intra-assay (n = 10)} & & & & \\
Mean & 7.6 & 14.4 & 24.3 & 52.9 \\
SD & 0.56 & 0.67 & 1.19 & 1.9 \\
CV, % & 7.4 & 4.6 & 4.9 & 3.5 \\
\textbf{Interassay (n = 29)} & & & & \\
Mean & 8.3 & 14.9 & 22.7 & 47.4 \\
SD & 0.82 & 0.94 & 1.44 & 3.5 \\
CV, % & 9.8 & 6.3 & 6.3 & 7.4 \\
\hline
\end{tabular}
\caption{Imprecision of FT\textsubscript{4} Measurement (pmol/L) in Serum Quality-Control Pools}
\end{table}
abscissa) being demonstrated up to a final sample dilution of 18-fold for euthyroid and thyrotoxic samples. The poorer linearity seen with hypothyroid samples is probably related to the fact that the measurements involved are close to the detection limit of the assay. A similar dilution experiment with serum from a euthyroid volunteer was incorporated into a time-course experiment; the results (Figure 4) suggested that linearity of response is maintained if incubation time is increased to 3 h. The effect of sample dilution and of microcapsule concentration is shown in Table 2. No significant change in measured FT₄ was apparent if diluted samples were assayed with double or half the optimized concentration of thyroxin-labeled magnetic microcapsules.

Comparison with external quality-control samples: Retrospective analysis of 15 samples from the U.K. External Quality Assessment Scheme for thyroid hormones gave FT₄ results (y) that compared well with the "all-laboratory trimmed mean" (x): y = 0.89x + 0.61 pmol/L (r = 0.99).

Diagnostic value of FT₄: In Table 3 are reported the concentrations of serum FT₄ determined for 165 healthy euthyroid adult volunteers; 54 clinically thyrotoxic patients in whom TT₄ and TT₃ were above our upper limits of normal and TSH was undetectable (<0.05 milli-int. unit/L); 50 clinically hypothyroid patients in whom TT₄ was less than our lower limit of normal and TSH was significantly increased (>15 milli-int. units/L); and 36 patients with suspected hypothyroidism with increased TSH (>15 milli-int. units/L) but TT₄ within the reference range (compensated euthyroidism). Results are also reported for serum FT₄ concentrations in a group (n = 54) of treated hypothyroid patients receiving between 0.05 and 0.30 mg of thyroxin daily, all of whom were clinically euthyroid and had serum TSH concentrations within the euthyroid reference interval. FT₄ concentrations obtained in euthyroid pregnant women during the first, second, and third trimesters are also included in Table 3.

To gain information on the practical implications of nonthyroidal illness on FT₄ measurement, we selected 38 serum samples from routine thyroid-function requests received in this laboratory within a one-week period. We selected samples from patients who had clinical evidence of nonthyroidal illness and had abnormal thyroid-function test results associated with such illnesses (i.e., TT₄ subnormal, TT₃ low normal or subnormal, and TSH normal). Seven of these patients had renal failure; the rest had various disorders, including heart failure, depression, flu, anemia, weight loss, myopathy, collapse, and jaundice. Their FT₄ concentrations are shown in Table 3.

Correlation of FT₄ with binding-protein concentration: There is no dependence of FT₄ on either the serum concentration of TBG or albumin with this assay (Figure 5).
modest increases in FT₄ results were, in all but one case, less than the lower limit of the thyrotoxic group (<24 pmol/L). This contrasts with grossly increased results obtained for the same specimens measured with an analog method (Amerlex-M; Amersham International).

Effects of adding oleic acid: The effect of oleic acid on the FT₄ concentration measured in serum from a euthyroid subject is shown in Figure 7. There was no significant increase in FT₄ until oleic acid >2.5 mmol/L was added.

Discussion

It is difficult to prove that an assay of FT₄ genuinely measures only the free fraction, and there has been much debate on the validity of several methods for this analyte (7, 8, 25). In an attempt to clarify the situation, Jackson and Ekins (26) suggested that for an FT₄ assay to be valid, it must possess a secure theoretical foundation and survive particular tests of analytical validity. The strongest support for the latter is maintenance of the measured concentration of FT₄ while thyroxin is released from TBG binding, when serum is diluted in an appropriate buffer (8).

A theoretical explanation for the membrane diffusion-controlled dialysis process that occurs in the microencapsulated procedure for FT₄ has been proposed by Buehler et al. (27). The mathematical model they describe for this kinetic system suggests that, if nonequilibrium measurement time is very short compared with equilibrium time, no significant perturbation of thyroxin/binding-proteins complexes occurs. Under these conditions, the measurement should be independent of the concentration of the protein-bound fraction within the sample. This is supported by our experimental findings that results obtained are independent of anti-T₄ circulating antibody, TBG, and albumin concentrations. Independence from binding proteins in serum has been reported for nonanalog (28) and some (29, 30), but not all (2, 7), analog methods. Buehler’s model predicts that an absolute requirement for independence from binding proteins in serum in a one-step nonanalog procedure is that the antibody must be retained within a semipermeable membrane. We must point out, however, that like many other mathematical models, the model described by Buehler et al. (27) is incomplete; it describes diffusion through the membrane but takes no account of the diffusion of labeled or unlabeled FT₄ within or outside of the microcapsule.

Our dilution experiment results indicate that samples from euthyroid and thyrotoxic subjects showed no significant change in the measured FT₄ concentration for a final
dilution of up to 18-fold. This range is wider than the 10-fold dilution reported for the Amerlex analog method (37) but narrower than the linearity of up to 100-fold dilution obtained with standard equilibrium dialysis procedures. Our results also show that sample dilution measurements are not altered by doubling the microcapsule concentration or significantly increasing the incubation time. This indicates that the optimized assay is well within limits that would not sequester an unacceptable high proportion of FT₄. Our results differ from findings with the Liquisol microcapsule FT₄ method, which showed a tendency towards higher results if the concentration of microcapsules was doubled during incubation with serum samples (32).

The euthyroid reference range for FT₄ in this study (8.4–18.4 pmol/L) is similar to that reported for the equilibrium dialysis methods of Helenius and Liewendahl (4) and Ellis and Ekins (3) (9–21 and 10–22 pmol/L, respectively), but its upper limit is lower than that suggested by Amersham International for the Amerlex method (9.4–25 pmol/L). As described, the standard material in this study, as in most others, has been calibrated against standards with concentrations validated by equilibrium dialysis. Furthermore, external quality-control FT₄ concentrations as measured by the microcapsule method agreed well with the all-laboratory trimmed mean. Strict comparison of published reference ranges could be misleading, given differences in the "normal" populations used. We established our reference range with a healthy, euthyroid, adult nonhospitalized Scottish population, but reference ranges can differ depending on the health of the euthyroid population (31, 33). Thus most kit manufacturers provide reference intervals but sensibly suggest that users should determine a range for the local population.

FT₄ results obtained with our microencapsulated assay during pregnancy indicate that values are similar to those found in nonpregnant controls. These findings are in agreement with results for the microencapsulated Liquisol method (2, 34, 35); most other methods, however, including equilibrium dialysis, indicate a small but significant drop below the lower limit of the nonpregnant reference range towards the end of pregnancy (26). We cannot explain why this drop is not apparent when FT₄ is measured by the microcapsule method, but in practice this finding suggests that there is no need to compile a separate normal range for pregnant subjects.

This method provided excellent discrimination between euthyroid controls and thyrotoxic patients during this study—at least in part because of a lack of interference from albumin, TBG, and circulating anti-T₄ autoantibodies. This last finding confirms an earlier report that interference by autoantibodies was eliminated by a microencapsulated system (36). Lack of TBG interference was found in some, but not all, batches of reagent used in the Liquisol microcapsule FT₄ method (29). The only situation with a possible discrimination problem at the upper limit of normal was with samples from patients with dysalbuminemic hyperthyroxinemia; in five out of seven such samples, results of the microencapsulated assay of FT₄ were modestly increased. Unlike the situation with some analog measurements (2), this increase was not great enough, in most cases, to place results in the thyrotoxic range; confusion in interpretation would be avoided by simultaneous TSH measurement. Discrimination between euthyroid controls and hypothyroid patients was also very good, though not absolute. Our results suggest that, if FT₄ results are considered in conjunction with TSH measurements, classification of patients as being either hypothyroid (and requiring thyroid replacement) or as compensated euthyroid (and requiring both clinical and biochemical follow-up) is possible.

In agreement with Pearce and Himsworth (37) we found normal or modestly increased FT₄ concentrations in clinically euthyroid patients receiving thyroxin. Excluded from our group, however, are patients with undetectable TSH concentrations (i.e., <0.05 milli-int. unit/L), a result thought by some to be a marker of over-replacement (38). We find it interesting that the FT₄ upper limit for the treated patients was similar to the lower limit for our untreated thyrotoxic group, which suggests that microencapsulated FT₄ measurements have a role in monitoring thyroid replacement. This area is currently under more detailed investigation.

Addition of the most common nonesterified free fatty acid, oleic acid, to normal serum did not increase measured FT₄ until the amount added was in excess of 2.5 mmol/L. Because the normal concentration of nonesterified fatty acids in serum is ~0.5 mmol/L, increasing to concentrations of up to 1.5 mmol/L in nonthyroidal illnesses (39), it is unlikely that they cause significant interferences in the microencapsulated FT₄ assay. This is in agreement with the findings of Lim et al. (40), who showed that addition of oleic acid at a concentration of 2 mmol/L did not release thyroxin from binding proteins but that release was significant at 3.5 mmol/L, with a doubling of the FT₄ fraction when the oleic acid concentration was 5 mmol/L.

Although thyroid-function tests are not recommended for patients suffering from nonthyroidal illnesses, most centers find that a significant proportion of routine requests are received from such patients. Our finding that approximately 10% of ill patients have modestly low FT₄ concentrations is in agreement with results obtained by an ultrafiltration reference method, but abnormalities are less frequent than the reported 35% abnormally low results obtained with analog methodology (39).

In 1986 Pearce and Byfield (41) stated that "a single thyroid function test which provides 100% diagnostically accurate information does not exist, nor is likely to." Our findings concur and we suggest that FT₄ measured by the microcapsule method, used in conjunction with a sensitive TSH assay, should provide a reliable indication of thyroid status.

Financial support was obtained from the Scottish Home and Health Department and Immunodiagnostics Systems Ltd., Usworth Hall, Washington, Tyne and Wear, U.K. We are indebted to many clinicians within Glasgow Royal Infirmary and to members of this Institute for help and advice; we are especially grateful to encouragement from Dr. D. O'Reilly. Thanks are also due to Dr. Wendy Ratcliffe, Wolfson Research Laboratories, Queen Elizabeth Medical Centre, Birmingham (for supplying serum from patients with anti-T₄ autoantibodies), and to Dr. P. Byfield, Clinical Research Centre, Northwick Park Hospital, London (for supplying some of the serum samples from patients with dysalbuminemic hyperthyroxinemia).

References


