LABORATORY OBSERVATIONS OF ASSAYS OF SERUM THYROXINE AND PROTEIN-BOUND IODINE

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Serum protein-bound iodine (PBI) has been used for a number of years as an indirect index in assessing thyroid function. The various methods used to determine PBI have been subject to chemical interferences which often rendered the final results doubtful or even invalid (*1*). Introduction of automation into the assays of PBI has minimized a number of procedural interferences and has improved the chemical accuracy and reproducibility of the final results (*2,3*).

Recently, a specific method for quantiting total serum thyroxine has been described by Murphy (*4*). According to this procedure the serum thyroxine is quantitated by isotope dilution using the properties of serum in competitive binding of labeled and unlabeled thyroxine.

We would like to report our laboratory experience on the routine determinations of serum thyroxine by competitive protein-binding analysis and by the chemical assay of PBI using a Technicon Auto-Analyzer.

METHODS AND MATERIALS

Serum thyroxine (T_4). Assays were carried out using the method described by Murphy (*4*) with some modifications which included the use of crystallir.e l-thyroxine sodium pentahydrate as a reference standard and a procedure for monitoring quality control.

Stable crystalline l-thyroxine sodium pentahydrate (Mann Research Laboratories Inc., 136 Liberty St., New York) was used to prepare the standard solution. The working solutions were made up in 70% ethanol equivalent to 5, 7.5, 10, 15 and 20 μg% of thyroxine using dilutions of a stock solution which contained an accurately weighed standard. Solutions, if not in use, were stored in a refrigerator and prepared fresh monthly. Radioactive ^{125}I-thyroxine (specific activity 30–75 mCi/mg) dissolved in 50% propylene glycol was purchased monthly from Abbott Laboratories. Radioactive reagent was prepared in 2,000-ml volumetric flasks in barbital buffer (pH 8.6, ionic strength 0.75) containing 20 ml propylene glycol, 100–120 μCi ^{125}I-thyroxine, 20 ml 1% aqueous phenol and 48 ml pooled human serum. The reagent was equilibrated at room temperature for 24 hr; if not in use, it was stored in the refrigerator. Fresh radioactive thyroxine solution was prepared every 2–3 weeks.

Strong base organic anion-exchange resin Rexyn-202 (mesh size 16-50) used for adsorption of free thyroxine was purchased in bulk from the Fisher Scientific Company. Before use the resin was soaked overnight in barbital buffer at room temperature and subsequently washed with distilled water. The purpose of this treatment was primarily the removal of soluble contaminants and floating resin particles. The washed resin was dried overnight at 100°C and stored in screwcap bottles.

Extraction of serum thyroxine. One milliliter of patient's serum was pipetted into a 15-ml centrifuge tube followed by 2 ml of 95% ethanol .The specimen was mixed rapidly with a stainless-steel wire. After the specimen was mixed for 30 sec the precipitated protein was centrifuged. From the resulting supernatant, 0.3 ml were transferred into each of three culture tubes and the solvent evaporated under a stream of air by heating the tubes in a water bath at 45 ± 1°C.

For quality control, pooled human serum and Moni-trol (Dade Co.) were treated the same way as the unknown specimens.

Thyroxine standards. With each set of 6–8 serum specimens a series of thyroxine standards was prepared. Three tenths milliliters of standard solution containing the equivalent of 0, 5, 7.5, 10, 15 and 20 μg% thyroxine were delivered into culture tubes in triplicate for each level. The solvents were evaporated at 45°C in the way described previously.

Equilibration of specimens with radioactive thyroxine. Using a calibrated automatic dispensing pipette 1.0 ml of the radioactive thyroxine reagent was added to each tube containing serum extract or thyroxine standard. The rack of tubes was gently

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shaken on a reciprocating shaker for 2 min at room temperature and then placed in a constant-temperature water bath and incubated for 8 min at 45 ± 1°C. After incubation the specimens were again gently shaken for 2 min and the rack was kept in a water bath at +4 ± 1°C for 10 min.

Separation of free and bound thyroxine. To each of the cooled specimens 0.5 ml of Rexyn-202 was added with a measuring dispenser. The tubes were gently shaken on a reciprocating shaker for 3 min and then placed in a water bath cooled with ice. Three milliliters of cold barbital buffer (pH 8.6) were added to each tube with calibrated repipet, and the specimens were gently shaken to insure complete mixing. From each tube 2.0 ml of supernatant containing bound thyroxine were pipetted into gamma-counting vials.

Determination of radioactivity. The ^{125}I activity was detected and quantitated with automatic gamma-counting systems (Nuclear-Chicago Model 4230 and Model 4216). Radioactivity detected in the specimen vials ranged from 24×10^3 cpm to 13×10^3 cpm. Each vial was counted for 2 min.

Calculation of thyroxine concentration. The average of radioactivity detected in the triplicates of thyroxine standards was plotted against the concentration of thyroxine expressed in $\mu g\%$.

In unknown specimens the average of triplicate counts was used to estimate thyroxine content from the standard curve, which is obtained with each set of specimens.

Serum protein-bound iodine (PBI). The serum total iodine and protein-bound iodine was determined by an AutoAnalyzer using the method described by Technicon (*2,3*).

RESULTS

Variables in serum thyroxine determinations. The assay of serum thyroxine (T_4) by the principle of competitive protein binding and the adsorption of free thyroxine by anion-exchange resin limits the number of specimens that can be processed in one batch by a single technician. By trial and error, we found that the T_4 assay has to be carried out in an arrangement with adequate quality-control monitoring.

Figure 1 shows a typical arrangement of specimens for a single set used in serum T_4 assay. The unknown serum specimens are preceded by a quality-control serum. Between the unknowns a previously assayed serum specimen is spaced as a double blind control and at the end of the unknown specimens another quality-control serum is included.

In an acceptable assay the results obtained with the quality-control specimens should not differ by more than 2 standard deviations (s.d.) of the mean.

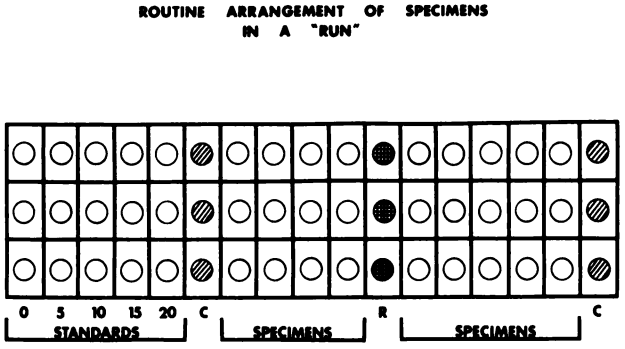

FIG. 1. Arrangement of specimens for a single set used in serum T_4 assay. Horizontal row is individual specimens. Vertical row is triplicate of same specimen. C is control serum and R is repeat of previously assayed serum specimen.

The coefficient of variation for serum thyroxine assay was verified. Serum specimens were assayed in duplicate and the standard deviation was calculated according to the following formula:

$$\text{s.d.} = \left[\frac{\text{sum (difference between duplicates)}^2}{2\text{ (number of pairs)}}\right]^{1/2}.$$

The coefficient of variation (c.v.) was calculated as percent using the expression:

$$\text{c.v.} = \frac{\text{s.d.}}{\text{mean value of results}}\,100.$$

Repeated determinations of serum thyroxine were made on specimens with high, normal and low concentrations of T_4. Results shown in Table 1 indicate that the coefficient of variation for the groups of high and normal T_4 is 5.2% and 5.4%, respectively. In the group of specimens showing low T_4 content, the coefficient of variation increases considerably.

Reproducibility of serum T_4 assays has been investigated using pooled human serum which was aliquoted in quantities of 5 ml and stored at −20°C. Table 2 reveals that the same pooled human serum assayed 14 times by three different technicians showed a mean T_4 concentration of 8.3 $\mu g\%$. The standard deviation of the mean in the case of each technician was very similar indeed. The effects of repeated thawing and freezing on the values of serum T_4 have not been investigated.

TABLE 1. ESTIMATION OF PRECISION FOR SERUM T_4 TEST

Serum T_4 levels	No. of pairs	Mean T_4 ($\mu g\%$ ± s.d.)	Coefficient of variation (c.v.)
High	14	12.0 ± 0.624	5.2%
Normal	87	7.5 ± 0.405	5.4%
Low	27	2.1 ± 0.390	18.6%

TABLE 2. REPRODUCIBILITY OF SERUM T_4 ASSAYS BY DIFFERENT TECHNOLOGISTS

Pooled human serum	Technologist	No. determinations	Mean T_4 (μg %)	1 s.d. (μg %)
#1	A	14	8.3	±0.5
#1	B	14	8.3	±0.4
#1	C	14	8.3	±0.5

For quality control in serum T_4 determinations, pooled human serum and commercial reference sera have been used. Moni-trol II (Lot #PTD 14 Dade Co.) with a high concentration of thyroxine had a mean T_4 of 13.6 μg% with a standard deviation ± 0.83 μg% in 17 individual assays. Moni-trol I (Lot #LTD 88 Dade Co.) assayed 23 times showed a mean thyroxine concentration of 7.3 μg% ± 0.41 μg% (1 s.d.).

To verify the normal range for this serum thyroxine assay method, the levels of T_4 were determined in 193 healthy male college students and government employees. The ages of these individuals ranged from 17 to 60 years. As Fig. 2 shows, this population sample revealed that only two subjects had T_4 below 4 μg% and one individual had T_4 above 11 μg%. Of all the serum specimens assayed 98% were found to be within the range from 4 to 10.5 μg%.

Protein-bound iodine (PBI). The reproducibility and quality control of the protein-bound-iodine determinations was monitored using Iodo-trol (Dade Co.) and pooled human serum. In 79 assays of Iodotrol reference substance the mean concentration of iodine was 8.56 μg% ± 0.21 (1 s.d.). Pooled human serum assayed 71 times showed a mean PBI of 6.60 μg% ± 0.27 (1 s.d.). The automated method for serum PBI determination revealed a better reproducibility statistically than the assay of serum thyroxine.

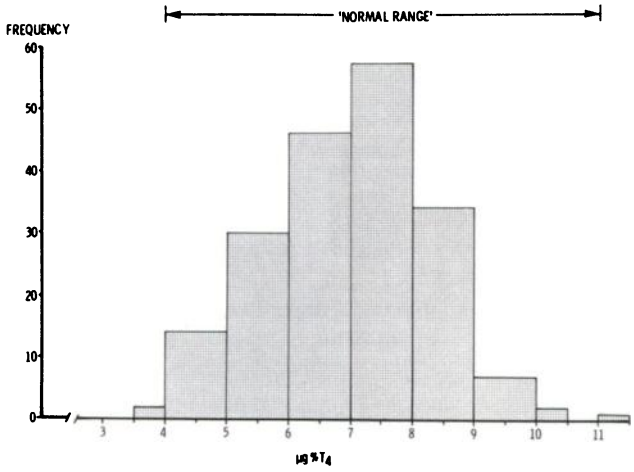

FIG. 2. Frequency distribution of serum thyroxine levels in healthy males.

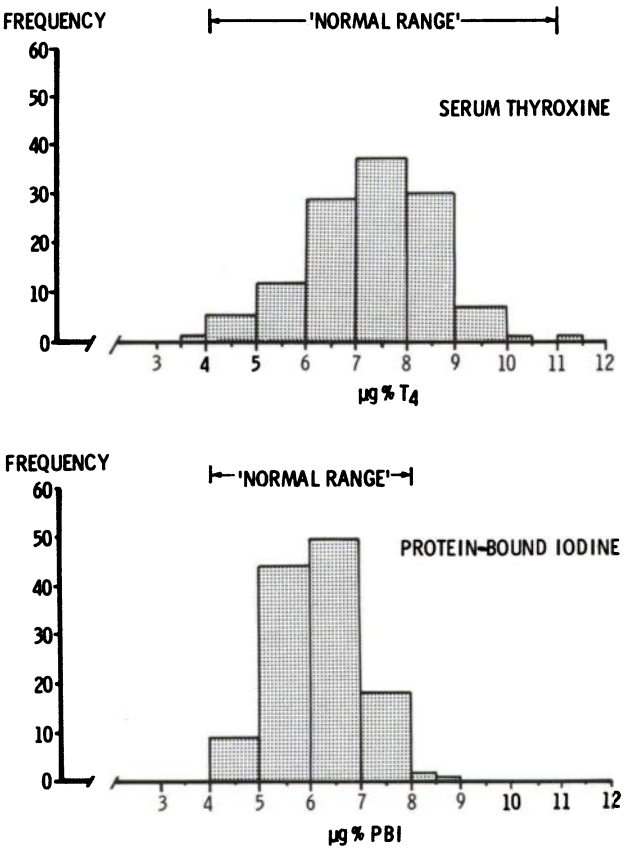

FIG. 3. Frequency distribution of serum PBI and serum thyroxine levels in healthy males.

The normal range of serum protein-bound iodine was compared with that of serum thyroxine. Simultaneous assays were performed using both methods on blood samples from 123 healthy males (age 17–60 years). Figure 3 shows the frequency distribution of PBI values and those of thyroxine. The PBI values from 4 to 8 μg% were found in 97% of the population.

Simultaneous assays of serum PBI and thyroxine have been performed on 220 individuals (148 females and 72 males) with suspected thyroid dysfunction. The results of these assays, graphically presented in Fig. 4, show a reasonable correlation. Because of different variables involved in this population, a regression line was not calculated. In this mixed population individuals appear who have PBI values within the "normal" limits whereas the serum thyroxine is below 4 μg%. A few individuals show protein-bound iodine above 8 μg% but the serum thyroxine falls within the "normal" limits. Diagnostic accuracy of serum T_4 and PBI methods as well as the discrepancies between these methods will be investigated at a future date as more laboratory results become available.

DISCUSSION

The method of serum thyroxine determination by competitive protein binding and adsorption of free thyroxine to anion exchange resin used in our laboratory produces results which are comparable with those of the original authors (*5*).

Modification of the technical procedures in serum T_4 assays has improved the reproducibility of the final results. Introduction of quality-control sera in the serum T_4 assay allows a statistical survey of the procedure and of the performance of the technician. Serum T_4 results on specimens with a normal thyroxine concentration and high thyroxine concentration show a similar coefficient of variation. The coefficient of variation, in our experience, increases considerably in serum specimens with low thyroxine concentration which is mainly due to the low mean value.

Apart from common variables in analytical procedures, the serum thyroxine assay has specific steps which can introduce technical errors. In this procedure the completeness of equilibration and the resin adsorption of the free thyroxine are the vulnerable ones.

The adsorption of thyroxine to Rexyn-202 depends not only on the time of contact of the resin with the solution, but also on the quality of the resin which varies from one manufacturers' lot to another. To minimize the latter variable, various lots of Rexyn-202 were tested and compared for their quality in adsorption of free thyroxine. The lot of Rexyn-202 which produced results within the limits of this study was applied for routine use.

Murphy *et al* (*5*) suggested normal limits for serum thyroxine concentration which range from 4 to 11 μg% which is applicable for males as well as females. To verify this "normal" range, the T_4 concentration was determined in 193 healthy males. Our results in this series indicate that 97.2% of all the individuals have serum T_4 concentration between 4 and 10 μg%. This observation is similar to that reported by the original authors of the method (*5*).

Investigations into the distribution of protein-bound-iodine levels in a population of 123 healthy males has revealed that 97% of the individuals had PBI in a range from 4 to 8 μg%. The same specimens assayed for T_4 concentration in 97.5% of the total population were found to be in a range from 4 to 10 μg%.

It is well recognized that the protein-bound-iodine method is not specific for thyroid hormones; nevertheless, the PBI values correlate reasonably well with the levels of serum thyroxine.

Technically the automated PBI method shows a reproducibility with a standard deviation which is considerably lower than that of the T_4 assay.

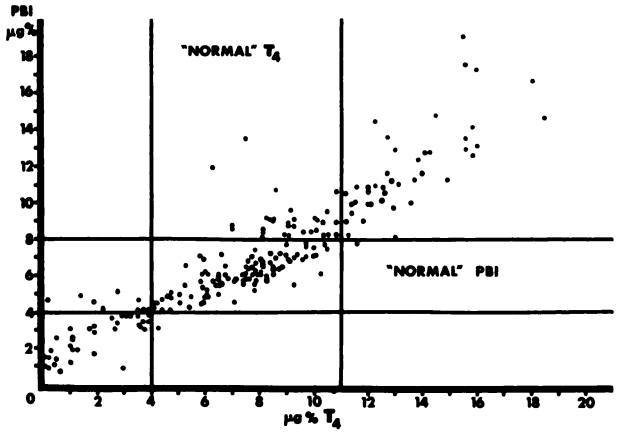

FIG. 4. Correlation of serum PBI with serum thyroxine in specimens assayed simultaneously using both methods.

Attention should be drawn to the fact that PBI assay is subject to interference. The most serious interferences in PBI values are from exposure of the patient to iodinated drugs or iodinated contrast media used in radiology. Excess iodine in the organism renders the PBI values completely invalid.

The presence of mercury in serum specimens interferes with the chemical determination of PBI, producing low results. As shown by Murphy *et al* (*5*) these factors do not effect the serum T_4 assay.

In the present study the degree of diagnostic accuracy of the serum T_4 was not specifically investigated. The original authors (*5*) of the method have reported that overlaps with the "normal" range do occur in the hypothyroid as well as in the hyperthyroid range.

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