CHAPTER II

MATERIALS AND METHODS

Patient Selection

Fifty two newly diagnosed Graves' disease patients were selected from the Out Patient Department of Ramathibodi Hospital for the study. The diagnosis was made on the basis of (1) elevation of serum T_4 by competitive protein binding, (2) increased 4 hour and 24 hour radioactive iodide uptakes, (3) diffuse and uniform uptake of ^{131}I on the thyroid scan, and (4) diffuse enlargement of the thyroid gland on physical examination without palpable nodularity. Five of the fifty two patients were male and the rest were female. Mean age of the whole group was 30.06 ± 10.12 and ranges from 16 to 58 years. The ages of the males range from 29 to 58 with the mean of 36.6 ± 12.54 years. The ages of the females range from 16 to 53 with the mean of 30.4 ± 10.16 years. All patients had never been treated with any mode of therapy for their thyroid diseases in the past.

After diagnosis was established blood was drawn for serum T_4, serum T_3, complete blood count, and liver function determination before initiation of therapy. After baseline blood was drawn all patients were given methimazole (Tapazole ®) one tablet (5 mg) every eight hours. Blood was drawn for the laboratory tests above at weekly intervals for 4 weeks, then at two months after therapy, and every two months thereafter until the end of the eighth month.

Only forty eight out of fifty two patients were used to analyse

incidence of T_3 toxicosis because serum T_3 values were not obtained in four patients in the first visit. Twenty three patients completed the weekly visits during the first month and they were used for analysis of data of serum T_4 and T_3 in the first month. At the time of analysis six patients had been followed for less than eight months; therefore only forty six patients were included in the analysis of longterm follow up for incidence of hypothyroidism with medication. Only fifty one patients were used for analysis of side effect of skin rash because one patient failed to have a follow up visit in the first month, the time with skin rashes occur in some other patients. Only 29 out of 33 hypothyroxinemia patients were used to analyse serum T_3 in hypothyroid state because four patients failed to have the value of serum T_3 in hypothyroid state.

Blood samples for the assay of normal values of serum T_4 and serum T_3 were obtained from the National Blood Centre, Thai Red Cross Society.

Determination of Total Serum Thyroxine

1. Materials

Sodium diethyl barbiturate (E. Merck)

Diethyl barbituric acid (Dallichipure Chemical)

Ethanol (Univar)

Radioactive l-thyroxine-5-5-Iodine-125 (The Radiochemical Centre, Amersham England)

Standard thyroxine (Sigma Chemical Company)

Propylene glycol (Vidhyasom Co.)

Resin beads (Amerite, IRA 400c, analytical grade, medium perosity)

Pooled pregnant serum

2. Instruments

Electrical balance (E. Mettler H. 20 T)

Centrifuge (International Centrifuge, size 2, Model K)

Gamma counter (Nuclear Chicago)

pH-meter (Hitachi, Horiba)

Mixer (Vortex Genic, Model K, 550 G.E.)

Pump (Arthur H. Thomas Co., Serial No. 62-71354)

Vanker variable speed rotator (Clay-Adams INC)

Hot-plate/Magnetic stirrer (Lapine Scientific Company, Cat. No. 834-75)

Waterbath (Thelco, Precision Scientific Co., Model 83)

Polystyrene tubes 17 x 100 mm., 10 x 75 mm.

Micropipettes 50, 100, 200, 300 µl (Eppendorf)

1 ml (Oxford Laboratory)

Refrigerator (Frigidaire)

Pipettor (Oxford Laboratories)

3. Preparation of barbital buffer solution

Barbital buffer solution was prepared by dissolving 15.45 g of sodium diethyl barbiturate and 2.76 g of diethyl barbituric acid in a 1-liter volumetric flask with a small amount of water and the volume was then made up with water to 1000 ml. The resultant solution was adjusted with barbital sodium or barbituric acid to pH 8.6.

4. Preparation of standard l-thyroxine solutions

4.1 Preparation of stock standard solution of l-thyroxine

(5 µg/ml). Five hundred micrograms of l-thyroxine were dissolved in 100 ml of 95% ethanol. This solution was stored in the freezer and was stable for at least one year.

4.2 Preparation of working standard solution of l-thyroxine. Eight-tenths of a milliliter of the stock standard solution (5 µg/ml) was diluted with 95% ethanol to 100 ml. The solution was stored in the freezer and was stable for several months.

5. Preparation of the standard TBG-^{125}I solution

One ml of propylene glycol and 1 ml of pooled pregnant serum were diluted with 98 ml of barbital buffer solution. T_4-^{125}I was added into this solution to give total count of 50,000 cpm/ml.

6. Preparation of resin beads

Resin beads were prepared for use by washing with distilled water in a shallow dish, and decanting the supernate several times to remove the "fines". The resin was then soaked overnight. After soaking it was washed several times with distilled water in a Buchner funnel which was fitted to a suction flask. After removal of as much water as possible by light suction, it was placed in a shallow glass dish and dried at not more than 60°C. The resin was stirred occasionally to accelerate the drying process which might take about 12 hours. If the resin was over-dried the beads were exposed in a shallow layer to the room atmosphere of low to medium humidity for a day or two. When properly prepared the beads could be poured freely and did not cling to the resin scoop.

7. Assay procedure (Modification of Murphy and Jachan, 1965)

7.1 Extraction of T_4 from the serum sample. Extraction was carried out at room temperature by adding 1 ml of each of the serum samples to 2 ml of ethanol in a polystyrene tube. The precipitate formed was immediately dispersed thoroughly by Vortex mixer for one minute. The tubes were stoppered and allowed to stand for 5 minutes. They were then centrifuged at 2,000 rpm for 4 minutes. Duplicated of 0.2 ml aliquots of the supernate were then placed in polystyrene tubes (17 x 100 mm.) for assay of T_4.

A pooled serum control or reference was carried along with each group of unknowns. The control tubes were placed at the beginning and at the end of each group of unknowns in order to get a good average value for the control.

7.2 Preparation of standard curve. Standard curve was prepared by pipetting aliquots of standard T_4 solution as in table below

ml of working standard solution	ng of T_4
0.0	0
0.05	2
0.1	4
0.2	8
0.3	12
0.4	16

Each of the above aliquot of working standard T_4 solution was placed in a polystyrene tube in duplicate to be assayed along with

the serum samples.

7.3 Evaporation. All tubes were placed in the water bath at 45°C and the evaporator was applied. The air current was gentle, and the evaporation was complete in 30 minutes.

7.4 Adding TBG-T_4-^{125}I. The tubes were removed from the bath as soon as evaporation was completed and to each was added 100 µl of TBG-T_4-^{125}I solution. Another pair of duplicates containing only the TBG-T_4-^{125}I was placed along with the above tubes to obtain "Total Counts".

All the tubes were shaken by Vortex mixer and placed in the refrigerator for at least 30 minutes.

7.5 Adding resin beads to separate bound T_4 from free T_4. After incubation for 30 minutes the tubes were removed from the refrigerator and placed in the ice bath. Half a ml of resin was added to each tube in quick succession. No resin was added to the pair of duplicates reserved for "Total Counts" (a funnel was used to prevent resin beads from clinging to the wall of the tubes). The tubes were then placed in the horizontal shaker for 30 minutes. After shaking, 3 ml of cold distilled water were added with sufficient force to form a uniform solution in each tube including the tube reserved for "Total Counts".

One milliliter aliquot of the supernate from each tube was placed in the counting tube for counting in the scintillation counter.

8. Calculation

8.1 The counts of the duplicates were summed and the percentage

of the "Total Counts" in the supernate was determined.

8.2 The standard curve was plotted with the percentage of "Total Counts" in the supernate as ordinate and ng of T_4 as abscissa (Fig. 2).

8.3 The T_4 of the unknown serum was read out from the curve. To obtain the T_4 in 100 ml serum, the value was multiplied by 1.5; the result was the value of T_4 in µg%.

The multiplication factor of 1.5 was used because aliquot of 0.2 ml of a total of 3 ml of extraction fluid was used for the assay, which resulted in the dilution factor of 15; and since the value obtained from the standard curve was in ng per ml and the value reported was per 100 ml, the total multiplication factor was then 1500 (value in ng%) or 1.5 (value in µg%).

Determination of Triiodothyronine

1. Materials

8-Anilino-1-naphthalene sulfonic acid (ANS) (Sigma Chemical Company)

T_3 Antibody (Rabbit plasma, Wein Laboratory)

Sodium diethyl barbiturate (E. Merck)

Diethyl barbituric acid (Dallichipure Chemical)

Bovine serum albumin (BSA) (Sigma Chemical Company)

Charcoal (Norit A, Fisher Chenical Co.)

Methyl cellulose (Union Chemical Co.)

Pooled serum (National Blood Centre, Thai Red Cross Society)

Sodium hydroxide (E. Merck)

Standard triiodothyronine (Sigma Chemical Company)

(3,3,5'-triiodo-l-thyronine sodium salt)

Radioactive triiodothyronine Iodine-125 (Liothyronine-I-125)

(Amersham)

2. Instruments

Electrical balance (E. Mettler, H. 20 T)

Centrifuge (International centrifuge, size 2, Model K)

Automatic refrigerated centrifuge (Damon/IEC Division, pr-6000)

Gamma counter (Nuclear Chicago)

pH-meter (Hitachi, Horiba)

Mixer (Vortex Genic, Model K, 550 G.E.)

Vantree variable speed rotator (Clay-Adams I.N.C.)

Hot plate-Magnetic stirrer (Lapine Scientific Company Cat. No. 834-76)

Water bath (Thelco, Pricision Scientific Co., Model 83)

Refrigerator (Frigidaire)

Micropipettes 100, 200, 300 µl (Eppendorf)

Pipettor (Oxford Laboratories)

3. Preparation of barbital buffer solution

(see section 3 page 12)

4. Preparation of T_3-free-serum

T_3-free serum was prepared as follows: Norit A charcoal was dried at 100°C for one hour. To 20 g of the dried charcoal in a tube, 100 ml of normal pooled serum were added. One drop of T_3-^{125}I was

also put into the mixture as indicator for completeness of absorption. The mixture was well shaken and then centrifuged at 2000 rpm. for 10 minutes. The tube was incubated at 4°C for 24 hours. After incubation, it was centrifuged again at 5000 rpm. for 30 minutes and the supernatant layer was collected. One drop of the supernate was then put into the gamma counter; if the count of the supernate was equal to the background count, it indicated that all T_3 in the pooled serum had been absorbed and that the T_3-free serum was then ready to be used in the determination of T_3. In case the count of the supernate was higher than that of the background, 10 g of dried charcoal were added to the supernate and the mixture was incubated at 4°C for 24 hours. It was then centrifuged and counted again. The procedure was repeated until the count of the supernate was equal to that of the background.

5. Preparation of charcoal-methyl cellulose mixture

A quarter of a gram of methyl cellulose was suspended in 100 ml of barbital buffer. Four grams of charcoal were placed in the beaker. The methyl cellulose suspension was added to charcoal, mixed and incubated at 4°C for 24 hours.

6. Preparation of standard triiodothyronine solution

A diluting solution was prepared by pipetting 2 ml of 20% propylene glycol into a 100-ml volumetric flask and then barbital buffer solution was added to 100 ml. The solution was used for diluting standard T_3.

6.1 Preparation of standard T_3 stock solution. One milligram of T_3 was dissolved in 1 ml of 0.01 M NaOH. and diluting solution was

added to make 100 ml solution.

6.2 Preparation of standard T_3 (10^{-5} g/100 ml). One ml of standard T_3 stock solution and 1 mg of bovine serum albumin were dissolved in 80 ml of diluting solution and additional diluting solution was added to 100 ml.

The solution was stored in the freezer and was stable for one month.

6.3 Preparation of standard T_3 working solution. Standard T_3 working solutions of different concentrations were prepared by diluting the standard T_3 (10^{-5} g/100 ml) solution as in the table below

T_3 concentration (pg/100 µl)	ml	ml of diluting solution
1000	1 ml of 10^{-5} g/100 ml solution	9.00
750	0.75 ml of 10^{-5} g/100 ml solution	9.25
500	5 ml of 1000 pg/100 µl solution	5.00
250	5 ml of 500 pg/100 µl solution	5.00
125	5 ml of 250 pg/100 µl solution	5.00
62.50	5 ml of 125 pg/100 µl solution	5.00
31.25	5 ml or 62.50 pg/100 µl solution	5.00

The standard solutions were stored in the freezer and were stable for one week.

7. Preparation of T_3-^{125}I solution

T_3-^{125}I solution was prepared by dissolving 15 pg of T_3-^{125}I in 0.1 ml of barbital buffer solution to give a total count of 10,000 cpm/ml.

8. Preparation of antibody solution

Antibody solution was prepared by adding barbital buffer solution to antibody so that antibody in 100 µl of antibody solution would bind approximately 30% of 37.5 pg of T_3. The solution was stored in the freezer and was stable for one month.

9. Preparation of 8-anilino-1-naphthalene sulfonic acid solution (ANS-solution)

Thirty mg of ANS were dissolved in 8 ml of barbital buffer solution and additional solution was added to make 10 ml.

10. Assay procedure (Fig. 1) (Modification of Mitsuma et al., 1971)

10.1 Three hundred microliters of barbital buffer solution were added to each tube intended for standard T_3 solution. Four hundred microliters of barbital buffer solution were added to the tubes reserved for "Total Counts" and "non specific binding of T_3" and "unknown". A hundred microliter T_3-free serum was added to all the tubes except the unknowns. A hundred microliter standard solution of each concentration was added to the standard tubes. A hundred microliter unknown serum was added to the tube "unknown". Reference serum was carried along with each group of unknowns as in the determination of serum T_4.

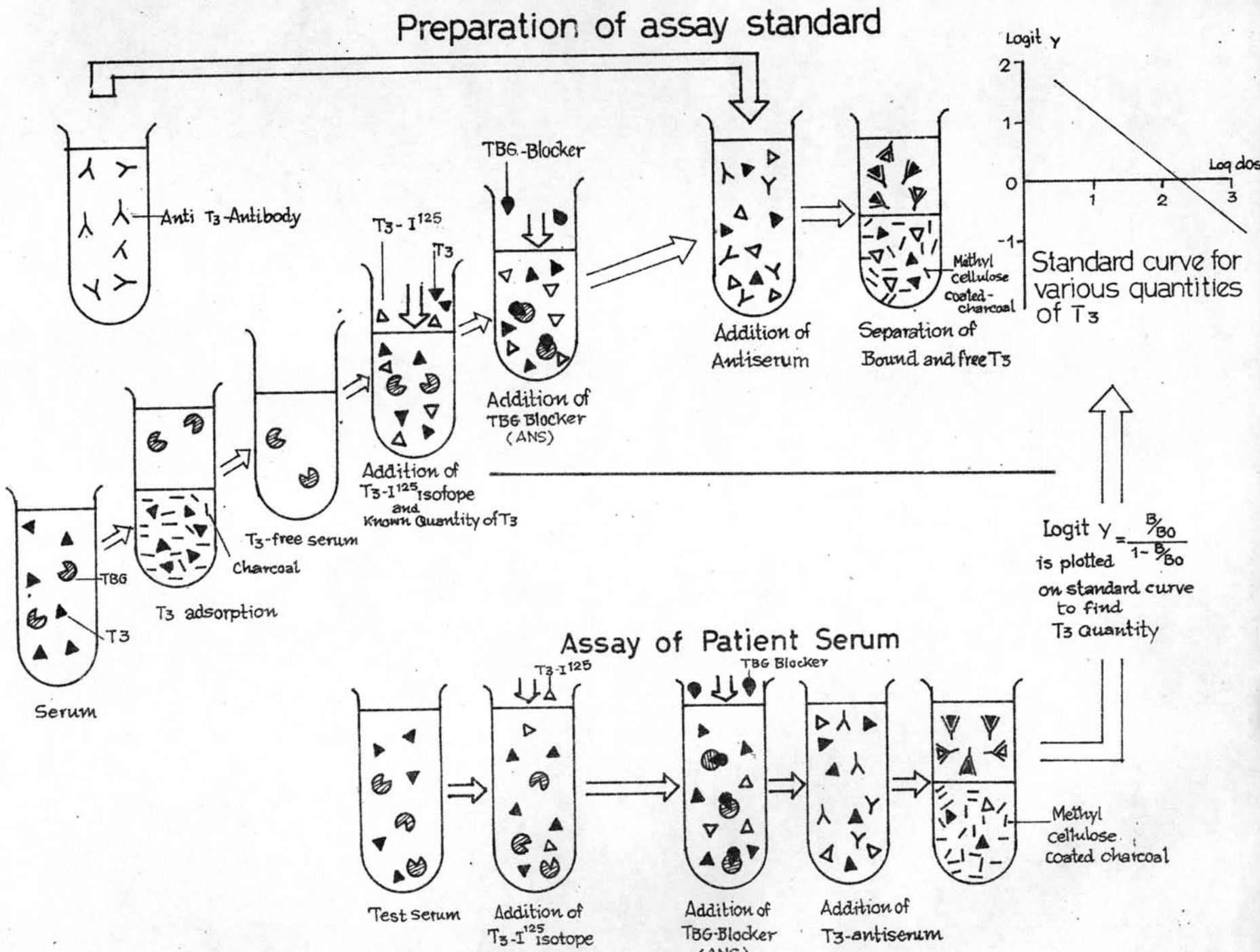

Fig. 1 Diagram of the T3 radioimmunoassay procedure

10.2 Blocking TBG A hundred microliter ANS solution was added to each of the tubes in quick succession. The tubes were shaken well and let stand for at least 10 minutes.

10.3 Adding T_3-^{125}I A hundred microliter T_3-^{125}I solution was added to all the tubes.

10.4 Adding Antibody A hundred microliter antibody solution was added to each tube. No antibody solution was added to the pair of the tubes reserved for "non specific binding of T_3".

All the tubes were shaken before incubation in the water bath at 37°C for 30 minutes. After incubation all tubes were removed from water bath and placed in the refrigerator for 24 hours.

10.5 Separation of the bound T_3 from free T_3 After incubation for 24 hours 0.2 ml of charcoal-methyl cellulose mixture was added to all tubes which were well shaken and let stand for 10 minutes. All tubes were centrifuged at 5,000 rpm for 30 minutes. Four-tenths of a milliliter aliquots of the supernate were pipetted into the counting tube for counting in the scintillation counter.

11. Calculation

11.1 The duplicate counts of the "non specific binding tubes" were averaged and the result was substracted from the counts of all other tubes.

$$\text{logit } B/B_o = \ln \frac{B/B_o}{1-B/B_o}$$

Where B_o is the count of "Total Counts".

and B is the count of unknown.

11.2 The standard curve was plotted with logit values as ordinate and log doses of T_3 as abcissa. (Fig. 3)

11.3 The serum T_3 was read out from the curve in log concentration of T_3. Value of antilog of this concentration was in pg/100 µl which is equal to ng/100 ml.