CHAPTER II

MATERIALS & METHODS

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Materials:-

A. Instruments:

1. Automatic Gamma Scintillation Spectrometer. (model 1280 Ultrogamma, LKB, Sweden).

2. Automatic refrigerated centrifuge. (Sorvall, RC-3, USA).

3. Refrigerator. (Philco ford, no frost 14, USA).

4. Water bath control temperature at 37°C. (Blue M, Electric Company, Blue Island, Illinois, USA).

5. Centrifuge. (Hettich EBA, Zentrifugen D-7200, 72 Tuttingen, Western Germany).

6. Blood volummertre. (model THC-3, Throcomp Elscint).

7. Calculator. (Olivetti programma 101).

8. Analytical balance. (type H-16, E.Mettler, Zürich, Switzerland).

9. pH-meter. (model 7030, Electric Instrument Ltd., Surrey, England).

10. Votex mixer. (Lab Line Instruments, Inc., Melrose park, Illinois, USA).

11. Rotator. (H.I.Clements & Son Pty. Ltd., Sydney, Australia).

12. Stir-plate & Mangetic bars. (Sybron Corporation, Nuova 7, USA).

13. Chromaflex column, 12 x 250 mm. (Kontes Glass Co., Vineland, N.J. 08360, USA).

14. Automatic pipettes; 25, 50, 100, 200, 300 & 500 ul. (Medical Laboratory Automation, Inc., Mount Vernon, N.Y.10550, USA).

15. Disposable syringes 10 ml and disposable needles No 20. (Terumo Corporation, Tokyo, Japan).

16. 12 x 75 mm, glass tubes for Radioimmunoassay. (Pyrex Laboratory Glassware).

17. Sero-pipettes; 0.5, 1, 2, 5 & 10 ml.

18. Hamilton microsyringes; 50, 100 ul. (Terumo Corporation, Tokyo, Japan).

19. Disposable pipette tips; 25-100 ul and 250-500 ul. (Medical Laboratory Automation, Inc., Mount Vernon, N.Y.10550, USA).

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B. Control and treated subjects:

1. Sera from normal, hypothyroid and hyperthyroid were obtained from the Nuclear Medicine Section, Department of Radiology, Siriraj Hospital. All sera were tested both in vivo and in vitro for euthyroid, hypothyroid and hyperthyroid subjects, respectively.

2. The sera of Acetaminophen treated were obtained from

the patients of Department of Surgery, Taksin Hospital, but some sera were obtained from healthy volunteers.

3. The sera of Diazepam treated were also supplied from the Department of Medicine, Taksin Hospital, but some sera were obtained from healthy volunteers.

4. The sera of Nordiol treated were kindly supplied from the Family Planning Research Unit, Department of Obstetrics & Gynaecology, Siriraj Hospital.

C. Experimental reagents:

1. For thyroxine radioimmunoassay.

1.1 Thyroxine-free serum was prepared by extracting serum T_4 from 100 ml pooled normal serum with 10 gm of unwashed Norit A charcoal (Sigma Chemical Corp., St.Louis, Missouri, USA) by mixing overnight on a rotator a 4°C. The charcoal was removed by centrifugation and filtration through millipores filters. This serum was stored a -20°C.

1.2 Standard T_4 for calibration standard curve. L-T_4 free acid (Sigma Chemical Corporation) 10 mg was dissolved in 2-3 drops of 0.1N NaOH, and then diluted to a concentration of 100 ugT_4 per one millilitre with 0.25% BSA in 0.06M barbital buffer, pH 8.6. One millilitre of this solution was aliquoted and stored a -20°C for future analysis.

1.3 0.06M Barbital buffer, pH 8.6: 20 gm of sodium

barbitone and 0.2 gm of sodium azide (Fisher Scientific Company and Chemical Manufacturing Division, Fair lawn, N.J., USA) were dissolved in 800 ml distilled water, adjusted to a pH of 8.6 with diluted hydrochloric acid and then made up to 1 litre with distilled water giving a 0.1M barbital buffer. For preparing a 0.06M barbital buffer, 600 ml of 0.1M barbital buffer was diluted to 1 litre with distilled water and the rest of it (400 ml) was diluted to 666 ml.

1.4 ^{125}I-T_4 in 75% ethanol with specific activity of 1200 uCi/ug (Radiochemical Centre, Amersham, England) was repurified on Sephadex G-75, fine column and eluted by the mixture of diluent buffer and 0.1M phosphate buffer, pH 7.6.

1.5 0.1M Phosphate buffer, pH 7.6: 13.92 gm of dibasic potassium phosphate, 2.76 gm of monobasic sodium phosphate and 8.76 gm of sodium chloride (E.Merck, Darmstadt, Germany) were dissolved in 800 ml distilled water and adjusted to a pH of 7.6 with diluted hydrochloric acid then made up to 1.0 litre with distilled water and stored at 4°C.

1.6 Diluent buffer, pH 7.4: 100 ml of 0.1M phosphate buffer, pH 7.6 was diluted with 800 ml distilled water and the 7.9 gm of sodium chloride and 10 gm of BSA were dissolved in this buffer and adjusted to a pH of 7.4. The volume was made up to 1 litre with distilled water and stored frozen.

1.7 Anti-T_4 antiserum or thyroxine antibody (Rabbit plasma, Lyophilised) was purchased from Wien Laboratories Inc.(Austria).

One vial was reconstituted with 5.2 ml of diluent buffer, pH 7.6 and then stored at 4°C.

1.8 8-anilino-1-naphthalene sulphonic acid (ANS): 90 mg of ANS (Sigma Chemical Company) was dissolved in 15 ml of 1% T_4-free serum in barbital buffer to give a concentration of 6 mg per one millilitre. Freshly prepared before use.

1.9 Polyethylene glycol (PEG): 20 gm of PEG (Sigma Chemical Company) was dissolved in 100 ml of 0.25% BSA in barbital buffer, mixed on a stir-plate until PEG was completely dissolved and stored at 4°C.

1.10 Coating buffer, pH 7.4: 7.9 gm of sodium chloride, 5 gm BSA and 3.8 gm disodium ethylenediaminetetra-acetate were dissolved in a mixture of 800 ml distilled water and 100 ml of 0.1M phosphate buffer, pH 7.6. The pH was adjusted to 7.4, made up to 1 litre with distilled water and stored at 4°C.

1.11 Sephadex G-75, fine (Pharmacia Fine Chemicals AB, Uppsala, Sweden) was soaked in 0.1M phosphate buffer, pH 7.6 overnight before packing the column.

2. For T_3-uptake test.

2.1 1N Phosphate buffer, pH 7.5 : 113.568 gm of disodium hydrogen phosphate was dissolved in 800 ml distilled water, and 41.403 gm sodium dihydrogen phosphate monohydrate (E.Merck, Darmstadt) was dissolved in 300 ml distilled water. These two solutions were

mixed together on a stir-plate and adjusted to a pH of 7.5 with diluted hydrochloric acid.

2.2 1/15N Phosphate buffer, pH 7.5 : 50 ml of 1N phosphat buffer, pH 7.5 was diluted in 700 ml distilled water, dissolved 0.1 gm sodium azide and mixed well.

2.3 0.1M Phosphate buffer saline, pH 7.6 : 3.6814 gm of sodium hydrogen phosphate, 8.766 gm of sodium chloride (E.Merck, Darmstadt) and 0.2 gm of sodium azide (Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, N.J., USA) were dissolved in 800 ml distilled water, adjusted to a pH of 7.6 with diluted hydrochloric acid and then made up to 1.0 litre with distilled water

2.4 $^{125}I-T_3$ in 75% ethanol with specific activity of 1200 uCi/ug (Radiochemical Centre, Amersham, England) was diluted to 1:10 with 0.5% BSA in phosphate buffer saline.

Methods:-

A. Subjects & Controls.

The effects of Acetaminophen, Diazepam and Nordiol in this study was divided into 3 experiments. Control sera were collected before treating the medicine.

Experiment No 1: 11 subjects from Department of Surgery and 51 healthy volunteers were treated orally with Acetaminophen, a dose of 3 gm daily for 5 days. After treatment blood samples were

taken from a cubital vein and the serum was separated and stored frozen at -20°C until analysed. Comparison with control serum of the same subject in the same assay was made. Experiment No 2: 10 neurosis subjects (new cases) from Department of Medicine and 42 healthy volunteers were administered orally with Diazepam, a dose of 7-9 mg daily for 5 days. The daily total dose was divided into 3 times. For neurosis patients, 2 mg of diazepam in the morning and in the evening and 5 mg before sleeping were received but for healthy volunteers, the daily dose was divided into 2 times; 2 mg in the morning and 5 mg before sleeping. After 5 days of treatment, 5 ml of blood samples were taken and serum was separated. Experiment No 3: 51 out patients from Family Planning Research Unit, Siriraj Hospital, were orally taken 1 tab of Nordiol daily for 1 month. Blood samples were collected after treatment. Throughout the investigation period the sera were kept frozen for future analysis.

B. Thyroxine Radioimmunoassay.

1. Allow all reagents to reach room temperature and mix well before using.

2. Coat the 12 x 75 mm glass tubes with coating buffer and label them in triplicate for standard curve and duplicate for unknown samples. One aliquote of T_4 standard (100 ug/ml) was thawed and diluted in thyroxine-free serum to give a dilution containing 20, 15, 10, 7.5, 5, 2.5, 1.25, 0.75, 0.5 and 0.25 ugT_4/100 ml, respectively.

3. Add to appropriate tubes in triplicate:-

a) 25 ul of thyroxine-free serum (0.00 concentration) and "NSB" tubes.

b) 25 ul of each concentration of T_4 standard.

c) 25 ul of each unknown serum in duplicate.

4. Add 50 ul of ^{125}I-T_4 to each tube and only 50 ul of ^{125}I-T_4 in triplicate for total radioactivity. Gently mix on a votex mixer.

5. Add 100 ul of 600 ug ANS to each tube and gently mix on a votex mixer.

6. Add 25 ul of T_4-antibody to each tube except "NSB" tubes and mix well.

7. Incubate all tubes at 37°C in water bath for 1 hour. The water level must be kept above that of the level of solution in the tubes.

8. Add 500 ml of PEG into each tube (the final concentration of PEG in each tube is 15%) and mix on a votex mixer (before adding PEG let all tubes to reach room temperature).

9. Centrifuge all tubes, except "total radioactivity" tubes in automatic refrigerated centrifuge at 3600 rpm for 50 minutes.

10. Immediately after centrifugation, decant the supernatant and carefully blot the lip of each tube with a small piece of filter papers.

11. Measurement the radioactivity of each tube in an auto-

matic gamma counter.

12. The calibration standard curve was constructed by plotting the percentage radioactive bound (B/B_0) against the T_4 concentrations on a semi-log paper and concentration of T_4 in unknown serum was read from the standard curve.

C. T_3-uptake test.

1. Add 5.5 ml of 1/15N phosphate buffer, pH 7.5 into 0.7 gm of Sephadex G-25, coarse and let the gel equilibrate for 16-18 hours (overnight) at 4°C or at room temperature for 3 hours.

2. 50 ul of diluted $^{125}I-T_3$ (1:10 with 0.5% BSA in 0.1M phosphate buffer saline, pH 7.6) was added into each tube of equilibrate Sephadex G-25.

3. Mix on a rotator for 15 minutes at room temperature (25°C).

4. 100 ul of standard serum and unknow samples in duplicate were added into each tube and mixed on a rotator for 30 minutes.

5. 2 ml of supernatant from each tube was pipetted into plastic tubes for counting the radioactivity.

6. Calculate T_3-uptake by using this equation:-

$$T_3\text{-uptake} = \frac{\text{count of unknow}}{\text{count of standard}} \times \text{K value of standard.}$$

D. <u>Free Thyroxine Index (FTI)</u>, was calculated by the following equation:-

$$\%FTI = \frac{\text{Total } T_4 \text{ (by RIA)}}{T_3\text{-uptake}} \times 100$$