CHAPTER II

MATERIALS AND METHODS

Patient selection

Patients with nephrotic syndrome

Thirty-three newly diagnosed nephrotic syndrome patients were selected from the Pediatric Ward of Ramathibodi Hospital for the study. Nine of thirty-three patients were female and twenty-four were male. Mean age of the whole group was 7.03 $\pm$ 3.25 years and ranged from 2 to 15 years. None of the patients had ever received steroid therapy for any other condition, nor was any patient known to have any abnormality of the hypothalamo-pituitary-adrenal axis. The patients were divided into two groups. One group of ten patients received oral prednisolone 2 mg/kg/day as single daily dose. The other group of twenty-three patients received oral prednisolone 4 mg/kg/day once every other day. The study was carried out after 2 months of therapy in all patients.

Patients with rheumatic heart disease

Fourteen newly diagnosed rheumatic heart disease patients were selected from the Pediatric Ward of Ramathibodi Hospital for the study. Six of fourteen patients were female and eight were male. Mean age of the whole group was 7.64 $\pm$ 2.06 years and ranged from 5 to 13 years. None of the patients had ever received steroid therapy for any other condition, nor was any patient known to have

any abnormality of the hypothalamo-pituitary-adrenal axis. The patients were divided into five groups. All patients received oral prednisolone every day in 3-4 divided doses for 30 days. The first group consisting of three patients received oral prednisolone 3 times of physiologic dosage. (The physiologic dosage of cortisol is 15-20 mg/m^2/24hr, equivalent to 3-4 mg of prednisolone/m^2/24hr) (Kenny and Preeyasombat, 1966; Bongiovanni, 1972). The second group consisting of four patients received oral prednisolone 7.5 times of physiologic dosage. The third group consisting of two patients received oral prednisolone 10 times of physiologic dosage. The fourth group consisting of three patients received oral prednisolone 12.5 times of physiologic dosage. The fifth group consisting of two patients received oral prednisolone 15 times of physiologic dosage.

Patients with rheumatoid arthritis

Ten newly diagnosed rheumatoid arthritis (five female and five male) patients were selected from the Pediatric Ward of Ramathibodi Hospital for the study. The mean age of the whole group was 8.8 $\pm$ 2.66 years and ranged from 6 to 13 years. None of the patients had ever received previous steroid therapy for any other condition, nor was any patient known to have any abnormality of the hypothalamo-pituitary-adrenal axis. The patients were divided into three groups. All patients received oral prednisolone approximately 5 times of physiologic dosage every day in 3-4 divided doses with various duration of therapy. The first group consisting

of three patients received oral prednisolone for 5 months of therapy. The second group consisting of three patients received oral prednisolone for 9 months. The third group consisting of four patients received oral prednisolone for 12 months.

The 24-hour urine and blood specimens were collected on the same day. Plasma cortisol levels were measured at least twice on the same day i.e. at 7 to 9 a.m. and at 3 to 4 p.m. The 24 hour urine specimens were used for 17-hydroxycorticosteroid (17-OHCS) and 17-ketosteroid (17-KS) determinations. Most of the study were carried out completely except in one patient with nephrotic syndrome who died of renal failure before the 24 hour urine collection had been accomplished.

The normal blood and 24 hour urine specimens were collected from normal children in one district of Mahasarakarm province under the permission and knowledge of their parents. None of these subjects were malnourished, ill, nor taking any kind of medicine during the study, they were allowed to have a normal diet and their usual daily physical activities.

Determination of plasma cortisol

Materials

1. Ethanol (Univar)
2. Standard cortisol (Sigma Chemical Co.)
3. Radioactive tritiated cortisol (The Radiochemical Centre, Amersham England)
4. Plasma for preparing corticosteroid binding globulin
5. Sodium hydroxide (BDH)
6. Boric acid (Rexall Drug Company Lot No. S 15061)
7. Concentrated hydrochloric acid (Matheson Coleman & Bell)
8. POPOP; 1,4-Bis-(5-Phenyloxazol-2-yl)-Benzene (Sigma Chemical Co.)
9. PPO; 2,5-Diphenyloxazole (Sigma Chemical Co.)
10. Methoxyethanol (Mallinckrodt)
11. Toluene (J.T.Baker Chemical Co.)
12. Charcoal (Norit 'A' Decolorizing Carbon, Fisher Scientific Co.)
13. Dextran T 70 (Pharmacia Fine Chemical AB Uppsala, Sweden)

Instruments

1. Liquid scintillation spectrometer (Packard Model 3320)
2. Electrical balance (E.Mettler H. 20 T)
3. Mixer (Vortex Genic, Model K, 550 G.E.)
4. Centrifuge (International Centrifuge, Size 2 Model K)
5. Pump (Arthur H. Thomas Co; Serial No. 62-71354)
6. Waterbath (Thelco, Precision Scientific Co., Model 83)

7. Hot-plate/Magnetic stirrer (Lapine Scientific Company, Cat. No. 834-76)
8. pH meter (Hitachi,Horiba)
9. Polystyrene tubes 17x100 mm
10. Micropipettes 100, 200 ul (Eppendorf) 1 ml (Oxford Laboratories)
11. Pipettor (Oxford Laboratories)
12. Deep Freezer - 20°C (Thermovac Industries Corp.)

Methods for preparations of reagents for the determination of plasma cortisol

1. Boric buffer solution

In a 1-liter volumetric flask 8.25 gm of boric acid and 2.70 gm of sodium hydroxide were dissolved with 500 ml of distilled water. Three ml of concentrated hydrochloric acid was added and the resulting solution was diluted to 1,000 ml with distilled water. The solution was adjusted with hydrochloric acid to pH 7.9.

2. Standard cortisol solution

2.1 Preparation of stock standard solution of cortisol (1 µg/ml) : One-tenth of a mg of cortisol was dissolved in 100 ml of absolute ethanol. This solution was stored in the freezer.

2.2 Preparation of working standard solution of cortisol (0.01 µg/ml): One ml of the stock standard solution (1 µg/ml) was diluted with absolute ethanol to 100 ml. The solution was stored in the freezer and was stable for several months.

3. Standard CBG H^3- F solution

Three-tenths of a ml of the plasma after Decadron suppression was diluted with boric buffer solution to 100 ml. H^3- F was added into this solution to give total count of 10,000 cpm/ml.

4. Charcoal reagent

Charcoal reagent was prepared by dissolving 1.25 gm of charcoal and 125 mg of Dextran in a 500 Erlenmeyer flask with a small amount of boric buffer and the volume was then made up with boric buffer to 500 ml. The reagent was stored in the freezer and was stable for one month.

5. Scintillation fluid

Three-tenths of a gm of POPOP and 10 gm of PPO were dissolved in a 2,000 ml Erlenmeyer flask with 600 ml of methoxyethanol and 1,000 ml of toluene. The fluid was stored in the freezer.

Assay procedure (Modification of Murphy, B.E.P, 1971)

Duplicate tubes were used throughout the experiment

1. One-tenth of a ml of the plasma sample was added to 1.9 ml of ethanol in a polystyrene tubes. The precipitate and the supernatant were mixed with Vortex mixer and centrifuged at 2,500 rpm for 10 minutes. Duplicate aliquots of 0.2 ml of the supernate were placed in polystyrene tubes for assay of cortisol.

2. Aliquots of standard cortisol solution were pipetted

in a polystyrene tube in duplicate as below

ml of standard solution	µg of cortisol
0.0	0
0.05	5
0.1	10
0.15	15
0.20	20
0.30	30

Standard cortisol solutions were placed in the empty tubes and assayed along with the plasma samples.

3. All tubes were placed in the water bath at 40°C for evaporation.

4. When the evaporation was completed, the tubes were removed from the water bath. One ml of CBG H^3- F was added to each tube and mixed. Another pair of duplicates containing only the CBG H^3- F was placed along with the above tubes to obtain total counts.

5. All tubes were incubated in the water bath at 40°C for 2 minutes.

6. After incubation the tubes were removed from the water bath and placed in the refrigerator for at least 10 minutes.

7. All tubes were removed from the refrigerator and placed in the ice bath. One ml of charcoal reagent was added to each tube and mixed. No charcoal reagent was added to the pair of duplicates reserved for total counts.

8. All tubes were centrifuged at 2,500 rpm for 10 minutes.

9. One ml aliquot of the supernate from each tube was placed in the counting vials which contained 10 ml of the scintillation fluid.

10. Each vial was thoroughly mixed and counted in the scintillation counter.

Calculation

The standard curve was plotted with the percentage of total counts in the supernate as ordinate and ug of cortisol as abscissa.

The cortisol of the unknown was read out from the curve.

Determination of 17-hydroxycorticosteroids

Materials

1. Ketodase (Warner Lambert Pharmaceutical Co.)
2. Acetic acid (Wako Pure Chemical Industries Ltd.)
3. Sodium acetate (Mallinckrodt)
4. Chloroform (BDH)
5. Sodium hydroxide (BDH)
6. Hydrochloric acid (Matheson Coleman & Bell)
7. Absolute ethanol (Univar)
8. Concentrated sulfuric acid (BDH)
9. Phenylhydrazine HCl (E.Merck)
10. Standard tetrahydrocortisone (THE) (Sigma Chemical Co.)

Instruments

1. Electrical balance (E. Mettler H. 20T)
2. Mixer (Vortex Genic, Model K, 550 G.E.)
3. Centrifuge (International Centrifuge, Size 2, Model K)
4. Pump (Arthur H. Thomas Co; Serial No. 62-71354)
5. Waterbath (Thelco, Precision Scientific Co ; Model 83)
6. Spectrophotometer (Coleman Junior II, Model 6/20)
7. Pipettor (Oxford Laboratories)
8. Micropipetting system (200, 1000 µl) with disposible plastic tips (Oxford Sampler Model Q, Oxford Laboratories)
9. Deep Freezer - 20°C (Frigidaire)
10. Hot plate (Kjeldahl Digesting Apparatus, Arthur H. Thomas Company)

Methods for preparations of reagents for the determination of 17-hydroxycorticosteroids

1. Acetic acid, 0.2 M

   In a 1-liter volumetric flask 11.4 ml of gracial acetic acid was added in distilled water and adjusted to 1000 ml.

2. Sodium acetate, 0.2 M

   In a 1-liter volumetric flask 16.4 gm of sodium acetate was dissolved in distilled water and adjusted to 1000 ml.

3. Sodium hydroxide, 0.1 N

   Two gm of NaOH was dissolved in 500 ml of distilled water.

4. Hydrochloric acid, 0.1 N

In a 500 ml volumetric flask 4.3 ml of concentrated hydrochloric acid was added in distilled water and adjusted to 500 ml.

5. Reagent blank for 17-OHCS

Three hundred and ten ml of concentrated sulfuric acid was mixed with 190 ml of distilled water.

6. Chloroform

Freshly distilled before use.

7. Phenylhydrazine - H_2SO_4 mixture

Eighty mg of phenylhydrazine was dissolved in 50 ml of reagent blank for 17-OHCS .

8. Standard THE solution

8.1 Stock standard solution (o.1gm/100ml)

One-tenth of a gm of THE was dissolved in 100 ml of absolute ethanol. This solution was stored at $4^{o}C$.

8.2 Working solution of THE (10 µg/0.1ml)

One ml of stock standard solution (0.1gm/100ml) was diluted to 10 ml with absolute ethanol to provide a solution of 10 µg/0.1 ml of THE .

Assay procedure (Modification of Glenn and Nelson, 1953)

1. Ten ml of the urine specimen was placed in a test tube and buffered by adding 0.75 ml of 0.2 M sodium acetate and 0.75 ml of 0.2 M acetic acid solutions.

This is mixed well. One and a half ml of Ketodase was added and the sample was mixed again. The urine was then incubated at 37°C for 24 hours.

2. This urine was extracted 3 times with 10 ml redistilled chloroform.
3. This extract was evaporated until the volume remained 10 ml.
4. The extract from step 3 was washed with 3 ml of 0.1 N NaOH and then with 3 ml of 0.1 N HCl.
5. The extract was evaporated to dryness in a 40°C water bath under air stream.
6. The dried extract was dissolved in 5.0 ml of absolute ethanol and duplicate aliquots of 2 ml each were pipetted into two empty tubes. One tube was used as the blank and the other for unknown.
7. Three ml of reagent blank for 17-OHCS was added to the blank and the solution was mixed and allowed to stand in a dark room overnight.
8. Three ml of phenyl hydrazine -H_2SO_4 mixture was added to the unknown and the solution was mixed and allowed to stand in a dark room overnight.
9. The samples were read in the Coleman spectrophotometer at 370, 410 and 450 nm against the reagent blank.

Calculation

The corrected optical density of the standard and sample were calculated using the following formula:

Corrected absorbance $= O.D_{410} - \frac{1}{2}(O.D_{370} + O.D_{450})$

milligrams of 17-OHCS/24 hour =

$$\frac{\text{Corrected absorbance of sample}}{\text{Corrected absorbance of standard}} \times \frac{\text{24 hr Volume (ml)}}{1{,}000} \times 5$$

Determination of 17-ketosteroids

Materials

1. m-Dinitrobenzene (m-DNB) specifically purified (BDH)
2. Potassium hydroxide (BDH)
3. 3 β-hydroxyandrost - 5 en-17-one (DHA) (Sigma Chemical Co.)
4. Sodium hydroxide (BDH)
5. Concentrated hydrochloric acid (Matheson Coleman & Bell)
6. Toluene (J.T. Baker Chemical Co.)
7. Absolute ethanol (Univar)
8. Chloroform (BDH)

Instruments

1. Electrical balance (E. Mettler H. 20T)
2. Mixer (Vortex Genic, Model K, 550 G.E.)
3. Centrifuge (International Centrifuge, Size 2, Model K)
4. Pump (Arthur H. Thomas Co; Serial No. 62-71354)
5. Waterbath (Thelco, Precision Scientific Co; Model 83)
6. Spectrophotometer (Coleman Junior II, Model 6/20)
7. Pipettor (Oxford Laboratories)
8. Micropipetting system (200, 1000 µl) with disposible plastic tips (Oxford Sampler Model Q, Oxford Laboratories)

9. Deep Freezer - 20°C (Thermovac Industries Corp.)
10. Hot plate (Kjeldahl Digesting Apparatus, Arthur H. Thomas Company)
11. Distillator (Isomantle Control Type ERN/1, Isopad Ltd; Borehamwood, Herts, England)

Methods for preparations of reagents for the determination of 17-ketosteroids

1. Standard DHA solution

   1.1 Stock standard solution One-tenth of a gm of DHA was dissolved in 100 ml of absolute ethanol. This solution was stored at 4°C.

   1.2 Working solution of DHA One ml of stock standard solution (0.1 gm/100 ml) was diluted to 10 ml with absolute ethanol to provide a solution of 10 µg/0.1ml of DHA.

2. Sodium hydroxide solution 10% w/v

   Ten gm of sodium hydroxide was dissolved in a 100 ml volumetric flask with distilled water which was then made up to a final volume of 100 ml.

3. Ethanolic m-Dinitrobenzene solution (2%)

   One gm of m-Dinitrobenzene was dissolved in 50 ml of absolute ethanol. The solution was stored in the refrigerator and was warmed to room temperature before use.

4. Potassium hydroxide solution, 5 N

This solution was prepared freshly before use. 1.4 gm of potassium hydroxide was dissolved in 5 ml of distilled water.

5. Chloroform

Freshly distilled before use.

Assay procedure (Modification of Glenn and Nelson, 1953)

1. Ten ml of the urine specimen was placed in a 100 ml glass tube and was hydrolyzed by adding 3 ml of concentrated hydrochloric acid. The solution was boiled on the hot plate for 20 minutes.
2. The hydrolyzed urine were removed from hot plate and allowed to stand for 30 minutes at room temperature.
3. The hydrolyzed urine was extracted 3 times with freshly distilled chloroform.
4. Combined chloroform extracts were washed with 3 ml of 10% NaOH and then followed with 3 ml of distilled water.
5. Washed extract was evaporated to dryness in a 40^{o}C water bath under air stream.
6. Two-tenths ml of 20 ug DHA and absolute ethanol were used as standard and blank respectively. They were treated concomitantly with the specimen under the same conditions.
7. The dried extracts were dissolved with 2 ml of absolute ethanol and duplicate 0.2 ml aliquots were pipetted into

two empty tubes.

8. Two-tenths ml each of the m-dinitrobenzene solution and 5 N KOH were added and the solution was mixed and allowed to stand in a dark cool place for 105 minutes.
9. Two ml of absolute ethanol was added to the solution and mixed.
10. The absorbance was then read in a spectrophotometer at 515 nm against the blank within 10 minutes.

Calculation

The corrected opitcal density of the standard and sample were calculated using the following formula:

milligrams of 17-ketosteroid conjugates/24hr =

$$\frac{\text{optical density of sample}}{\text{optical density of standard}} \times \frac{\text{24 hr volume (ml)}}{1{,}000} \times 20$$