CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Subjects

1.1 Normal subjects. Blood sample of 100 blood donors, 50 male, and 50 female obtained from the Thai Red Cross Society, Bangkok were used as normal subjects.

1.2 People exposed to organophosphate insecticide

1.2.1 Blood samples of 58 workers from an insecticide factory were studied. 36 subjects had a history of the direct exposure to the insecticide and 22 subjects who work in the office do not contact the insecticide directly.

1.2.2 Blood sample of 11 patients who took organophosphate and carbamate insecticide accidentally or as a suicide were obtained from the department of Forensic Medicine, Chulalongkorn hospital.

1.3 Patients with various diseases

1.3.1 Blood samples of 124 patients with various diseases from the hospital for Tropical Disease, Faculty of Tropical Medicine were studied. They were comprised of 35 patients with infectious hepatitis, 57 patients with Plasmodium falciparum malaria.

1.3.2 Blood samples of 65 patients were obtained from the Department of Pediatric, Ramathibody Hospital. They were 30 patients with β.E thalassemia, 8 patients with Hb-H thalassemia, and 17 patients with congenital heart disease.

1.4 Pregnant women and Cord blood

1.4.1 Blood samples of 30 pregnant women in the first, second and third trimester were obtained from Siriraj Hospital.

1.4.2 Blood samples of 27 newborn infants were obtained from Siriraj Hospital.

2. Chemicals

2.1 5:5 dithiobis-(2-nitrobenzoic) acid or DTNB (Sigma Chemical Company)

2.2 Acetylthiocholine iodide (Sigma Chemical Company)

2.3 Tris (hydroxymethyl) aminomethane (Tris) (BDH, Analar grade)

2.4 Glutathione (reduced form) (Sigma Chemical Company)

2.5 Hydrochloric acid (E. Merck)

2.6 Sodium chloride (E. Merck)

2.7 Quinidine sulfate (Sigma Chemical Company)

2.8 Sodium bicarbonate (E. Merck)

2.9 Bovine erythrocyte cholinesterase (Sigma Chemical Company) 2.3 unit/mg (unit of the enzyme activity was expressed as micro mole of substrate hydrolyzed per min. per ml. at 37°C)

2.10 Ethylene diaminetetraacetic acid (May & Baker)

2.11 Disodium hydrogen phosphate (May & Baker)

2.12 Monopotassium hydrogen phosphate (May & Baker)

3. Glasswares

3.1 Volumetric flask, size 10, 25, 100, 250, 500 ml (Pyrex, Kimax)

3.2 Measuring cylinders, size 100 (Pyrex)

3.3 Test tube size 13 x 100 mm. (Pyrex, Kimax)

3.4 Test tube with screw cap size 16 x 100 mm. (Pyrex)

3.5 Automatic pipets (Adjustable volume) size 1-100 ml., 1-5 ml. (Gilson)

3.6 Automatic pipets (Adjustable volume) size 0.1-1 ml. (Clay-Adams)

3.7 Silica cuvett (Unicam, Beckman)

3.8 Heparinized Capillary tube (Jinton Terumo Co., Ltd.)

4. Instruments

4.1 Spectrophotometer (Unicam sp. 1800) equipped with a Unicam recorder

4.2 Ultrathermostated circulators (NB-36126-Colora)

4.3 Standard pH Meter (Radiometer, PHM 62)

4.4 Analytical balance (Harvard trip balance, Ohaus)

4.5 Electrical balance (E. Mettler, Type H 30)

4.6 Centrifuge (IFE Clinical)

4.7 Micro Hematocrit (Heraeus-Christ) with Hematocrit reader chart (Heraeus-Chirst)

4.8 Strip charts (Unicam)

METHODS

1. Determination of acetylcholinesterase (AChE) activity in the whole blood.

AChE acitivty in the whole blood was determined by the method described by Ellman et al (1961).

1.1 Preparation of blood samples

Blood specimens were drown from the vein and collected in the vial contained ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The specimens were kept at 4^{o}C. until assayed.

1.2 Determination of the haematocrit level

Two heparinized capillary tubes were filled with blood to a level of about two-thirds of the tubes. The dry end of the tube was sealed with the clay and centrifuged at 14,000 rpm in a micro-haematocrit centrifuge for 5 minutes. The haematocrit level was measured by using a haematocrit reader chart. The haematocrite value gives the number of millimeters of packed red cells per 100 millimeters blood which indicates the volume (%) of packed red cells per 100 ml blood.

1.3 Preparations of reagents

1.3.1 Phosphate buffer solution 0.1 M, pH 8.0

500 ml of 0.1 M, pH 8.0 phosphate buffer was prepared

by mixing 475 ml of 0.1 M Na_2HPO_4 (14.2 g per litre) and 25 ml of 0.1 M KH_2PO_4 (13.6 g/litre). The pH was adjusted to 8 by adding 0.1 M KH_2PO_4 solution.

1.3.2 Phosphate buffer solution 0.1 M, pH 7.0

10 ml of 0.1 M, pH 7.0 phosphate buffer was obtained by adding 0.1 M Na_2HPO_4 (14.2 g/litre) to 4 ml of 0.1 M KH_2PO_4 (13.6 g/litre). The pH was adjusted to 7 with few drops of 0.1 M Na_2HPO_4 solution.

1.3.3 Substrate Acetylthiocholine iodide, 0.075 M

21.67 mg of acetylthiocholine iodide was dissolved in 1 ml of distilled water. This solution can be used successfully for 10-15 days if kept in refrigerator.

1.3.4 Reagent Dithiobisnitrobenzoic acid (DTNB) 0.01 M

39.6 mg of DTNB was dissolved in 10 ml of phosphate buffer 0.1 M, pH 7.0 and 15 mg of sodium bicarbonate.

1.3.5 Enzyme, Bovine erythrocyte cholinesterase

10 mg (23 unit) of Bovine erythrocyte cholinesterase was dissolved in 10.0 ml of distilled water.

1.4 Preparation of a standard acetylcholinesterase activity curve.

The following standard solution of enzyme acetylcholinesterase was prepared by diluting 1.0 ml of 2.3 unit/ml of Bovine erythrocyte cholinesterase with distilled water.

AChE activity (Unit)	ml of standard AChE (2.3 unit/ml)	ml of water added
2.300	1.0	0.0
1.530	1.0	0.5
1.150	1.0	1.0
0.575	1.0	3.0

1.5 Methods for preparing the standard curve.

1. Exactly 3.0 ml of phosphate buffer (pH 8) were pipetted into two cuvettes, one for the test standard sample, and the other for the bank.

2. 100.0 µl of the DTNB reagent and 20.0 µl of substrate were added to both cuvettes and placed in the photometer.

3. The slit of the photometer was adjusted so that the absorbance (at 412 mµ) of the suspension in the cuvettes was zero.

4. 50.0 µl of the enzyme were added to the sample cuvette, and mixed by pipetting.

5. The change in absorbance at 412 mµ was recorded and the slope is the measurement of the rate in absorbance unit/min.

6. Standard curve of enzyme acetylcholinesterase activity was prepared by plotting the rate in absorbance unit/min against the unit of the standard enzyme.

1.6 Determination of acetylcholinesterase activity in whole blood

1. A 20 µl sample of blood was added to 12 ml buffer pH 8 and mixed by pipetting.

2. Exactly 3.0 ml of the suspension of blood from 1 were pipetted into a cuvette.

3. 25 µl of the DTNB reagent were added and the cuvette was placed in the photometer.

4. The slit of the photometer was adjusted so that the absorbance (at 412 mµ) of the suspension of blood from 3 in the cuvette was zero.

5. A 20 µl of substrated added to this cuvette.

6. The change in absorbance at 412 mµ was recorded at least 6 min.

Note:

1. The blank was consisted of 3.0 ml of the suspension and 25 µl of DTNB reagent. It is necessary to correct for release of thiol material from the cells and the absorbance of the other material in the suspension.

2. All samples were run in duplicate.

3. At this pH level, there is an appreciable non-enzymatic hydrolysis of the substrate, and for long runs it was necessary to correct for this error.

2. Study on serum cholinesterase in serum

Cholinesterase in serum was determined by the method described by Garry and Routh (1965).

2.1 Preparation of serum samples.

Blood specimen were drawn from the vein of subjects. Serum was seperated and stored at $-4^{o}C$ until assayed.

2.2 Preparation of reagents

2.2.1 DTNB buffer 25 mg of 5:5 dithiobis (2-nitrobenzoic acid), 1.66 gm. NaCl, 62.5 ml. 0.2 M tris (hydroxymethyl) aminomethane (Tris) were added into 100 ml. of 0.1 N HCl in a 250 ml. volumetric flask. The final volume was adjusted with distilled water. This reagent has a pH of 7.40 at 37°C and an ionic strength of 0.153. It is stable for 2 weeks if kept refrigerated.

2.2.2 Acetylthiocholine iodide 0.01 M was prepared to a final concentration of 5.2 mg/ml in distilled water. This solution (the substrate) is stable for 1 week if kept refrigerated.

2.2.3 Quinidine sulfate was prepared to 0.5% (w/v).

2.2.4 Glutathione stock solution. 25 mg of glutathione was dissolved in 100 ml of distilled water. It is equivalent to 8.13×10^{-4} M.

2.3 Preparation of a standard cholinesterase activity curve (Glutathione standard curve).

The glutathione standard curve was prepared from a stock solution containing 25 mg. glutathione in 100 ml. water. The following standard solutions were prepared.

Glutathione stock solution 25 mg/100 ml	DTNB buffer (ml)	Distilled water(ml)	Sulfhydryl group liberated (M)
0	4	1.5	0
0.1	4	1.5	8.13×10^{-4}
0.2	4	1.5	16.26×10^{-4}
0.3	4	1.5	24.39×10^{-4}
0.4	4	1.5	32.52×10^{-4}
0.5	4	1.5	40.65×10^{-4}

Method for standard

Aliquot of 0.1, 0.2, 0.3 0.4 and 0.5 ml of the stock solution of glutathione were added to 4.0 ml of the DTNB-buffer reagent. The mixtures were diluted with distilled water to a final volume of 5.5 ml, mixed by inversion, and the absorbance was measured at 412 mμ. One tube containing no glutathione was used as the blank, and its absorbance was subtracted from that of the standard.

2.4 Determination of serum cholinesterase activity.

Two 13 x 100 mm tubes were reguired for each determination, one for the test sample and the other for the blank. 4.0 ml. of the DTNB-buffer was pipetted into each tube and allowed the contents to equilibrate to 37° in a water bath. 20 μl of serum was added to both tubes followed by 1.0 ml. of 0.5% Quinidine solution to blank tubes. Then 0.5 ml. of the substrate solution was added to both tubes and mixed by inverting the tubes once. Exactly 3 min after the addition of the substrate, the enzyme reaction was stopped in the test sample by the addition of 1.0 ml. of inhibitor solution. The tube was removed from the waterbath and measured the absorbance at 412 mμ. The readings for each pair of tubes (blank and test sample) should be made within 1 min of each other.

CALCULATION

1. Calculation for whole blood cholinesterase activity

The cholinesterase activity is expressed as μM of substrate hydrolyzed/min/ml of blood at 37°C.

micromole substrate hydrolyzed/min/ml = $\Delta A \times 600 \times K \times 1.0411$

where ΔA = The difference in absorbance between the test sample and the blank.

600 = dilution factor

K = 1/Slope of the standard curve = $\frac{\text{enzyme units}}{\Delta A}$

1.0411 = correction factor of the volume of the solution.

2. Calculation of serum cholinesterase activity

The cholinesterase activity is expressed as micromoles of sulfhydryl groups liberated/min/ml of serum. The serum cholinesterase activity is then calculated as follows:

$$\mu\text{MSH/min/ml of serum} = \frac{\Delta A}{3} \times k \times 50$$

ΔA = the difference in absorbance between the test sample and the blank

50 = dilution factor

3 = time for enzyme reaction

and k is obtained from the glutathione calibration curve, which is equivalent to μM Glutathione/Absorbance unit.

3. Calculation of erythrocyte cholinesterase activity

The activity of erythrocyte cholinesterase was calculated using the following equations:

$$\text{RBC} = \frac{\text{Whole blood activity} - (1 - \text{hematocrit}/100)\ (\text{Serum activity})}{\text{hematocrit}/100}$$