CHAPTER III

MATERIALS AND METHODS
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A total of 121 Organophosphorus poisoning cases, aged 14-72 years with a male to female ratio of 2:1 admitted to Civil Hospital, Ahmedabad were included in this study. Detailed information of each case was recorded on a proforma which included (a) personal, family and past history regarding age, sex, address, occupation, socioeconomic status, education, marital status, psychological problems, major illness, past hospitalization, family disturbances. A detailed occupational history was taken from cases coming from industries. (b) History of present illness at the time of admission i.e. presenting complaints and symptoms observed during hospitalization etc. In suicidal attempts/accidental cases characterization of the agents was attempted on the basis of a container shown by the patient or family members. (c) The clinical examination as per the proforma consists of general and systemic examination including neurological examination, respiratory, CVS (including ECG recording) and GIT. (d) Investigations of the case.

The following laboratory investigations were carried out. A sample of venous blood was obtained from each individual.

Routine Investigations

(i) Haemogram

- Haemoglobin estimation - By Sahli's haemoglobinometer.
- Total and differential leucocyte count.
- Erythrocyte Sedimentation rate (ESR). – Wintrobe Method
Specific Investigations

1. Cholinesterase enzyme activity in erythrocytes and plasma were measured in all the cases at the time of admission in the hospital.

2. Renal function tests - Blood urea (Diacetyl Monoxime, WHO 1986), serum creatinine (Jaffe Reaction, WHO 1986), and electrolytes Na+ & K+ (Flame Emission Spectrometry - "Flame Photometer") were done in all cases.

3. Liver function tests – Serum bilirubin, serum glutamate pyruvate transaminase (SGPT) / Alanine aminotransferase (ALT), Serum glutamate oxaloacetate transaminase (SGOT) / Aspartate aminotransferase (AST) and Alkaline phosphatase were performed in all the cases.

4. Lactate Dehydrogenase (LDH) activity in serum was estimated in all cases.

5. Creatine Kinase (CK) activity was determined in 46 cases which included 10 controls.

6. Immunoglobulins such as IgG, IgA, and IgM, C3 and C4 complements were also measured in 40 cases which included 19 controls.

Diagnosis

The clinical diagnosis of Organophosphorus poisoning was made on the basis of a corroborative evidence of history of pesticide exposure, the symptoms and signs typical of anticholinesterase illness, inhibition of ChE activity and the presence of atropine refractoriness.
Reagent

1. 10 mg. of 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) was dissolved in 50 ml of 0.9% sodium chloride and was added to 50 ml of 1/15 M Sorenson phosphate buffer pH 8.0. This solution was stored in refrigerator.

2. 75 mg. of propionyl thiocholine was dissolved in 50 ml of distilled water. Fresh solution was prepared each day and kept in ice bath during the day’s work.

3. 50 mg. eserine salicylate was dissolved in 50 ml of distilled water. This solution was also stored in refrigerator.

Procedure

10 ml. of pre cooled DTNB/buffer solution was pipetted into a 15 ml. Centrifuge tube (tube no.1) and 10 microliter. of freshly collected whole blood was carefully added to it. Before 4 ml. of this solution was pipetted into another centrifuge tube (tube no. 2) and 1 ml. of substrate solution of propionylthiocholine was added. Immediately thereafter the stopwatch was started and the tube was placed in a constant water bath (30 °C) for exactly 10 min. 2 drops of eserine salicylate was added and mixed Tube no. 1 and no. 2 were then centrifuged for 5 min. at 3000 rpm. Before determination of absorbance of the yellow supernatent in tube no. 2 the klett Summerson colorimeter (blue filter 420 nm) was brought to zero with a mixture of 4 ml. of DTNB buffer and 1 ml of substrate solution. The supernatent solution in tube no. 2 was carefully decanted into the colorimeter without stirring up
For the standard and for each patient specimen pipetted 1.0 ml of caffeine-benzoate reagent into each of the two test tubes. One tube was used for standard or serum blank and one for the test.

Added 100 microlitre of standard or patient serum to each pair of tubes.

Added 0.5 ml of diazo reagent to the test and 0.5 ml of sulfanilic acid to the blank.

Mixed well and let stand for 10 minutes at room temperature.

Added 1.0 ml of alkaline tartrate reagent to each tube and mixed thoroughly.

Read the absorbance at 600 nm (Ilford filter No. 607) immediately setting the spectrometer to zero with distilled water.

Calculations

Calculated the results from linear calibration graph by using the following formula:

Concentration of bilirubin (micro mol/liter) = \[ \frac{T - TB}{S - SB} \times 342 \]

Where

\[ T \] = Absorbance reading of sample or control

\[ TB \] = Absorbance reading of control or patient sample blank.

\[ S \] = Absorbance reading of bilirubin standard (342 micro mol/liter)

\[ SB \] = Absorbance reading of standard blank

b) Serum Glutamate Pyruvate Transaminase (SGPT) / Alanine Aminotransferase (ALT)

Principle

Alaline aminotransferase catalyses the reaction.

\[ \text{L-Alaline} + 2-\text{Oxoglutarate} \xrightarrow{\text{GPT}} \text{Pyruvate} + \text{L Glutamate} \]

The product pyruvate formed oxidises NADH in the coupled enzymatic reaction:
Pyruvate + NAD + H → Lactate + NAD +

This reaction is followed spectrophotometrically by measuring the disappearance of absorbance due to NADH at 340 nm with time.

**Technique**

Prepared a reagent mixture by mixing phosphate alanine solution, 30 volume, Nicotinamide Adenine Dinucleotide (NADH) solution, 0.5 volume and LDH solution 0.5 volume (stable for 12 hours at 25 degree centigrade) and incubated at 25 degree centigrade. Pipetted into the cuvettes 3.1 ml reagent mixture and 0.5 ml serum sample, mixed and incubated for 5 min. Added 100 ml 2-oxoglutarate solution, mixed and followed the reaction (at 25 degree centigrade) at 340 nm for 5–10 min.

**Calculation**

Alanine aminotransferase activity (U/l) = 1190 x A340 nm/min.

c) Serum Glutamate Oxaloacetate Transaminase (SGOT) / Aspartate Aminotransferase (AST). Method: Colorimetric.

**Principle**

The enzyme catalyses the following reaction

\[
\text{ASAT} \quad \text{Aspartate} + \text{Alpha - Ketoglutarate} \rightarrow \text{Oxaloacetate} + \text{Glutamate}
\]

In the colorimetric procedure the oxaloacetate formed is converted to pyruvate and measured colorimetrically. Pyridoxal – 5 – phosphate is the coenzyme.

**Technique**

Added 9.8 ml glass distilled water to 0.2 ml of RBC (washed and stored) and kept for 1 hour.

Carried out duplicate analysis with all samples. Taken 0.5 ml of hemolysate in three sets of tubes (blank, basal activity and stimulated activity). Added 0.1 ml of
PLP solution to one set of tubes (stimulated activity) and incubated all the tubes at 37 degree centigrade for 30 minutes, in a shaker water bath shaking at low speed.

Added glass distilled water to all the tubes to make the volume to 1 ml. Added two drops of 100 % TCA solution to blank tubes. Added 0.5 ml buffered substrate solution to all the tubes. Incubated all the tubes at 37 degree centigrade for exactly 30 minutes. Added two drops of 100 % TCA to all the tubes except the blank tubes.

Added two drops of alaline citrate to all the tubes. Shaken well and left at room temperature for 20 min. Added 0.5 ml DNP solution. Mixed well and left at room temperature for 5 min. Added 2 ml toluene, shaken well and centrifuged at 2000 rpm for 5 min.

Transferred 1 ml toluene layer to clean dry tubes. Added 3 ml of alcoholic KOH and mixed thoroughly. Measure OD at 510 nm against a blank containing 1 ml toluene and 3 ml alcoholic KOH solution.

Ran a set of pyruvate standards (10-100 micro g) similarly. Plotted the absorbance against the concentrations.

Calculation

Subtracted the blank reading from the test and obtain the pyruvate content in each tube from the standard graph.

Enzyme activity = Microgm pyruvic acid x $\frac{10}{88.07}$ x $\frac{1}{0.5}$ x $\frac{60}{0.2}$ micro moles pyruvate/mi RBC/hr

Activation coefficient = $\frac{\text{Stimulated activity}}{\text{Basal activity}}$
d) Alkaline phosphatase. Method: King and Armstrong

*Principle*

\[ \text{Alkaline phosphatase} \]

\[ \text{p-Nitrophenyl phosphate} + \text{H}_2\text{O} \rightarrow \text{Nitrophenol} + \text{H}_3\text{PO}_4 \]

The amount of p-nitrophenol liberated in unit time, as determined in alkaline pH at 400-420 nm is a measure of the phosphatase activity.

*Technique*

Pipetted into tubes buffer substrate solution (pH 9.8), 2.0 ml and 50 micro litre serum. Mixed and incubated for exactly 30 minutes at 25 degree centigrade. Stopped the reaction by adding 10 ml 0.05 N NaOH. For blanks the sample is added after the NaOH addition. Read the absorbance at 405 nm against the respective blanks.

*Calculation*

Alkaline phosphatase activity (U/l) = \[ \frac{\Delta A_{405} \times 12.05 \times 1000}{18.5 \times 0.05} \]

*Lactate Dehydrogenase (LDH)*

LDH was assayed using kinetic method as described by the committee on Enzymes of the Scandinavian Society of Clinical Chemistry (1974).

*Principle*

The method is based on the conversion of pyruvate to lactate by lactate dehydrogenase using coenzyme NADH. The change (decrease) in extinction at 340 nm is used for calculation. The reaction is carried out at 37 °C and activity is expressed as U/L at 37 °C.

\[ \text{Pyruvate} + \text{NAD} + \text{H} \leftrightarrow \text{Lactate} + \text{NAD} \]
Reagent 1     NADH
Reagent 1A    Buffer
Reagent 2     Pyruvate
Mix Reagent 1 and 1A = Solution 1
Working solution: 10 volumes of solution 1 + 1 volume of solution 2
1 ml of working solution + 30 micro l sample, incubation temperature 37 ° C.
Read extinction at 340 nm.
E/min x 5520 = u/l

Creatine Kinase (CK)
The kinetic method was used for assay of creatine kinase.

Principle
Creatine Phosphate + ADP $\xrightarrow{CK}$ Creatine + ATP

Glucose + ATP $\xrightarrow{HK}$ G-6-P + ADP

G-6-P + NADP $\xrightarrow{G-6-PD}$ 6 - Phosphogluconate + NADPH + H+

Increase in the extinction due to NADPH formation is related to CK activity.
The N-acetyl Cysteine (NAC) is used as activator.

Reagents (1) Buffer Imidazole PH 6.7 0.1 mol/l
Glucose 20 mmol
Mg Acetate 10 mmol
EDTA 2 mmol
Sodium Azide 0.1
Reagents (2) ADP 2.0 mmol
AMP 5.0 mmol
Diadenosinepenta phosphate 10 micro mol
NADP 2.0 mmol
HK 2.5 IU/ml
G6 PDH 1.5 IU/ml
N-acetylcystein 20 mmol
Creatine phosphate 30 mmol

Working solution by reconstituting the volume given on the reagent bottle 1 & 2.

Working solution 1.0 ml
Sample 0.02 ml
Reaction temperature 37 °C

The extinction is measured at 340 nm.

Extinction per minute at 37 degree C x 8095 = IU/l.

**Immunoglobulins**

Solugen R plan i.e SRID plates (Immuno diagnostics pvt. Ltd., Delhi – 110 006, India) were used for analysis of Immunoglobulins IgG, IgA and IgM & also C3,C4 complements. These plates are based on Radial immuno-diffusion technique (Mancini et al., 1965). The sample (5 micro l) was charged with the help of micro pipette into the well of the plates. The plates were kept for incubation for 50 hrs. The diameters of precipitation ring (Ag – Ab, reaction) was measured after 50 hrs. using immuno measure Rx supplied by the manufacturer, after referring to the linear to log scale the Immunoglobulins and complement levels were obtained.