# CHAPTER II

# MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Biological Materials

Normal human sera were separated from red blood cells by centrifugation at 3,000 xg for 10 min , of whole blood obtained freshly from healthy donors.

Patient sera were collected at Institute of Occupational and Environmental Medicine , Rajvitee Hospital, from Pb toxic patients whose blood Pb level was higher than 30 $\mu$g/l , preserved in ice and immediately transferred to the laboratory. These sera were kept at -70°C until used, normally not more than 2 months.

Heparinized whole blood was obtained freshly from healthy donors and used within 1 hr.

Human transferrin and other standard proteins : alcohol dehydrogenase (bakers yeast) , bovine albumin, ovalbumin and human myoglobin were perchased from Sigma chemical company.

### 2.1.2 Chemicals

#### 2.1.2.1 Ferrozine Method

L - Ascorbic acid was purchased from BDH. Thiosemicarbazide was product of Merck chemical. 3-(2-Pyridyl)5,6-diphenyl-1,2,4 triazine- 4',4''-disulphonic acid monosodium salt (Ferrozine) was from Fluka and glycine was obtained from Sigma.

#### 2.1.2.2 Graphite Furnace Atomic Absorption Spectrophotometer (F-AAS) and Inductively Coupled Plasma Spectrophotometer (ICPS)

Standard Pb and iron were purchased from Normex farmitaria carlo erba. Triton X-100 was from Packard. All chemicals mentioned above were of atomic absorption grade.

#### 2.1.2.3 Chromatography

Sephadex G-25, Sephadex G-200 and blue dextran were products of Pharmacia Fine Chemicals. Potassium dichromate and sodium acetate were purchased from BDH. Lead acetate was from Mallinokrodt chemical company and glacial acetic acid was from Merck.

2.1.2.4 Discontinuous Polyacrylamide Gel Electrophoresis (Disc-PAGE) and Isoelectric Focusing Polyacrylamide Gel Electrophresis (IEF-PAGE)

Acrylamide (ultragrade) and N,N'-methylene-bis-acrylamide for IEF-PAGE were purchased from LKB , those for Disc-PAGE were products of Merck and Biorad. N,N,N',N'-Tetraethylmethylenediamine and bromophenol blue were from BDH. Ampholine pH 5-7 and ammonium persulfate were obtained from Pharmacia and Biorad. Coomassie brilliant blue R-250 was from Sigma, glacial acetic acid and methanol were from Merck.

2.1.2.5 Determination of Protein

Sodium hydroxide was from Eka Nobel Ltd. Copper sulfate and sodium carbonate were Merck Chemicals and sodium potassium tartrate was obtained from BDH.

2.1.2.6 Other Chemicals

Heparin was Leo pharmaceutical product. Tris-(hydroxymethyl)-aminomethane and imidazole were purchased from Fluka. Disodium hydrogen phosphate , monosodium dihydrogen phosphate and hydrochloric acid were from Merck. Monocalcium disodium EDTA , dimercaprol and D-pennicillamine were kindly provided by Institute of Occupational and Environmental Medicine.

2.1.3 Instruments

- Kokusan H-103N Series Centrifuge
- High Speed Microcentrifuge, model MC-15A
- Midget Electrophoresis Unit , model LKB 2050, LKB produkter AB, Sweden
- Fraction Collector , Pharmacia LKB Redi Frac, Pharmacia, Sweden
- LYPH - LOCK Freeze Dry System , model 77400, Labconnco
- Incubator, Memmert
- PHM 83 Autocal pH Meter, Radiometer, Copenhagen, Denmark
- Power Supply , model 1000/500, Bio - rad Laboratories, U.S.A
- Spectrometer, Spectronic 2000, Baush & Lomb

## 2.2 METHODS

### 2.2.1 Determination of Metal Concentration

Fe and Pb concentrations were determined by Graphite Furnace Atomic Absorption Spectrophotometer (F-AAS) or Inductively Coupled Plasma Spectrometer (ICPS). In some experiments , transferrin - bound iron was determined chemically with ferrozine reaction. The sample subjected to each method was prepared by different methods as described below.

Deionized water was used throughout the investigations. Glasswares and storage containers were soaked overnight with 1 M $HNO_3$ and rinsed with deionized water before used.

#### 2.2.1.1 Graphite Furnace Atomic Absorption Spectrophotometer (F-AAS)

##### 2.2.1.1.1 Sample Preparation

Sample containing high protein concentration but low metal level , such as serum , must be completely hydrolyzed before determining metal concentration by F-AAS. The hydrolyzing process was as described by Ohchi (Ohchi et al, 1985). One milliter of serum was dried and carbonized in crucible on a hot plate at 150°C for 3-4 hrs. and subsequently ashed at 500°C for 24 hrs. in a muffle

furnace. At hr 12 of the ashing process , the crucible was allowed to cool and 10 $\mu$l of 30 % $H_2O_2$ and 10$\mu$l of conc. $HNO_3$ were added. Afterwhich the temperature was gradually increased to 500°C and the process was continued for 12 hrs. As the ashing process was completed , the ash was dissolved in 1 ml of 0.02M $HNO_3$ and 16 $\mu$l of the solution was injected to F-AAS for metal concentration determination.

Sample containing high protein concentration (> 2 mg/ml) and high Pb level (> 1,800 $\mu$g/l) , such as Pb-treated plasma and red blood cells , need no hydrolysis. Such samples could be diluted 20 folds with 0.02 M $HNO_3$ and injected directly to F-AAS. Red blood cells were completely lysed with equal volume of 0.02 % Triton X-100 and centrifuged at 3,000 xg for 30 min. The supernatant obtained was diluted 20 folds with 0.02 M $HNO_3$. Sixteen microliters of the solution was injected to F-AAS for the determination of metal concentration.

Solutions of low protein concentration concentration (<2 mg/ml) such as fractions from column chromatography, could be injected directly to the F-AAS.

2.2.1.1.2 Spectrophotometer Settings

A spectrophotometer was operated at 283.3 nm for Pb determination and 248.3 nm for Fe determination, in the peak height mode with a 0.5 nm slit width , internal standards were included. The sample size for injection was

16 $\mu$l, and 0.1 M $H_3PO_4$ was used as emulsifier for Pb determination. The graphite-furnace heating variables and gas flow rates were shown in the following table.

| STEP NO. | TEMPERATURE °C | TIME (sec) | GAS FLOW (l/min) |
|---|---|---|---|
| 1 | 85 | 5.0 | 3.0 |
| 2 | 120 | 30.3 | 3.0 |
| 3 | 500 | 5.0 | 3.0 |
| 4 | 500 | 10.0 | 3.0 |
| 5 | 500 | 2.0 | 0.0 |
| 6 | 2100 | 0.8 | 0.0 |
| 7 | 2100 | 2.0 | 0.0 |
| 8 | 2600 | 5.0 | 3.0 |

### 2.2.1.2 Inductively Coupled Plasma Spectrometer(ICPS)

#### 2.2.1.2.1 Sample Preparation

The samples subjected to ICPS were fractions from Sephadex G-200 column chromatography , which contained less than 2 mg/ml protein. The samples of 2 ml were thus directly passed through the ICPS.

2.2.1.2.2 Spectrometer Settings

The ICPS measurement was made on a Perkin-Elmer Plasma 100 sequential emission spectrometer. The system was controlled by an IBM AT 386 computer which was used in delveloping the method, in acquiring and storing the data. A standard torch and Perkin-Elmer cross flow nebulizer (controlled by a peristaltic pump working at a flow rate of 1 ml $min^{-1}$) were used during the experiment. The operating conditions for ICPS instrument was shown in the following table.

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| | |
|---|---|
| Radiofrequency power | |
| Forward | 1,000 W |
| Reflected | <5 W |
| Gas flow rates | |
| Collant | 15 $min^{-1}$ |
| Auxiliary | 1 $min^{-1}$ |
| Nebulizer | 1 $min^{-1}$ |
| Observed wavelengths | |
| Pb | 220.353 nm |
| Fe | 259.940 nm |

Observation height : 15 mm above load coil

Peristaltic pump flow rate : 1 ml $min^{-1}$

Integration time : 1,000 ms / step

read delay : 20 s

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2.2.1.3 Iron Determination with Ferrozine Method (Mori, 1981)

Free iron concentration could be determined with Ferrozine method. Ferrozine and free iron form a magenta complex which is measured by the absorbance at 592 nm. Since most of the iron in serum is bound to transferrin, iron must be dissociated prior to Ferrozine reaction. The dissociation occurred at acid pH, with a reducing agent. The value of serum iron concentration determined can, therefore, represent transferrin-bound iron. The procedures for Fe determination with ferrozine are described below.

2.2.1.3.1 Reagents

a) Color Reagent

1 g of Ferrozine, 1 g of thiosemicarbazide and 6.25 ml of concentrated hydrochloric acid (conc. HCl) were dissolved in a one-liter volumetric flask two-thirds filled with water. The solution was slowly added with 30 ml of Triton X-100, mixed well until dissolved and adjusted to 1 l with distilled water. Just before used , 10 ml of this solution was added with 0.2 g of ascorbic acid.

Blank reagent was also prepared as color reagent but without Ferrozine.

b) Glycine Buffer

15.02 g of glycine was dissolved and adjusted to 1 l with 5 mM HCl.

2.2.1.3.2 Sample Preparation

0.5 ml of serum was diluted 3 folds with 0.2 M glycine buffer (b). The mixture was let stood for 15 min before adding 180 mg of magnesium carbonate power to remove free Fe. Then it was shaked continuously for 30 min and centrifuged at 3,000 xg for 5 min. The supernatant obtained was measured for iron concentration by ferrozine method (2.2.1.3.3).

2.2.1.3.3 Ferrozine Method

The following method for Fe concentration determination was described by Mori (1981). One milliliter of the sample was added into 2 ml of color reagent. The mixture was let stood for 10 min before measuring for $A_{562}$ which could be calculated for iron concentration from standard curve (0-100 mg/ml).

2.2.2 Gel Filtration Chromatography

2.2.2.1 Sephadex G-25 Column

The column (1 x 20 cm) was used for quick separation of free Pb from serum proteins. It was equilibrated and operated in 0.01 M acetate buffer, pH 4.15, at a flow rate of 20 ml/hr , sample size and fraction volume were 1 ml each. In checking the column resolution , cytochrome C and potassium dichromate were used.

2.2.2.2 Sephadex G-200 Column

This column was used not only to isolate free Pb from proteins but also to fractionate the serum proteins prior to metal and protein determination. A Sephadex G-200 column (1.8 x 120 cm) was equilibrated and operated in 0.01 M acetate buffer, pH 4.15, at a flow rate of 20 ml/hr. The sample size and fraction volume were 2 ml each. $A_{280}$ was measured in each fraction, 1 ml from each of them was drawn for metal determination with F-AAS, 3 ml volume was used in case of ICPS.

## 2.2.3 Discontinuous Polyacrylamide Gel Electrophoresis (Disc-PAGE)

### 2.2.3.1 Compositions and Stock Solutions

a) 30 % Acrylamide, 0.9 % Bis-acrylamide

29.1 g of acrylamide and 0.9 g of bis-acrylamide was dissolved in water , the volume was adjusted to 100 ml, filtered and stored in the dark bottle at 4 °C.

b) 0.05 M Tris-glycine Electrode Buffer, pH 8.3

6.0 g of Tris and 28.8 g of glycine was dissolved in 800 ml of distilled water, adjusted pH to 8.3 with 1 N HCl and the volume was made up to 1 l with distilled water.

c) 3.0 M Tris-HCl, pH 8.9

36.6 g of Tris, 0.3 ml of TEMED, and 15 ml of 6 N HCl were mixed, adjusted pH to 8.9 with 1 N HCl. The final

volume was made up to 100 ml with distilled water.

d) 0.5 M Tris-HCl, pH 6.7

5.98 g of Tris and 0.46 ml of TEMED were dissolved in 90 ml of distilled water, adjusted pH to 6.7 with 1 N HCl. The volume was made up to 100 ml with distilled water.

e) 10 % Ammonium Persulfate Solution

0.1 g of ammonium persulfate was dissolved in 1 ml of distilled water, prepared immediately before used.

f) Sample Buffer

20 ml of glycerol, 10 ml of stock solution (d) and 20 ml of distilled water were mixed well with 1 ml of 0.1 % of bromophenol blue.

g) Protein Staining Solution

0.5 g of coomassie blue R-250 was dissolved in 100 ml of methanol, stirred for one hour and filterd. Before used , 100 ml of this solution was mixed with 100 ml of 20 % acetic acid.

h) Protein Destaining Solution

First Destaining Solution:

The solution contained 10 % acetic acid and 50 % methanol.

Second Destaining Solution:

The solution contained 7 % acetic acid and 5 % methanol.

2.2.3.2 Preparation of Disc-PAGE

The separating gel (10 x 6 x 0.075 cm) containing 7.5 % acrylamide , and stacking gel (10 x 2 x 0.075 cm) were polymerized between two glass plates (10 x 8 cm) of Midget Electrophoresis Unit. The gels were prepared from stock solutions, as follow.

| Solutions | Separating gel 7.5 % T (ml) | Stacking gel 3 % T (ml) |
|---|---|---|
| 30 % Acrylamide solution | 5.00 | 1.0 |
| 3.0 M Tris-HCl stock solution, pH 8.9 | 2.50 | - |
| 0.5 M Tris-HCl stock solution, pH 6.7 | - | 1.25 |
| Distilled water | 12.5 | 7.75 |
| 10 % ammonium persulfate (μl) | 150 | 150 |
| Total volume (ml) | 20 | 10 |

2.2.3.3 Sample Preparation

Usually protein sample was suspended in sample buffer to give approximately 2.5 to 5 mg/ml concentrations about 4 $\mu$l was for electrophoresis application. In case of very diluted protein solution , as those fractions collected from Sephadex G-200 column , 100 $\mu$l of each fractions was lyophilized with freeze dry system and then dissolved in 10 $\mu$l of 0.01 M acetate buffer pH 4.15. 2 $\mu$l of this solution was then mixed with 2 $\mu$l of sample buffer and finally applied to polyacrylamide gel.

2.2.3.4 Electrophoresis Run

The electrophoresis was carried out vertically at 4°C in midget electrophoresis unit with a constant current (20 mA per gel) until the tracking dye came down to 0.5 cm from the bottom of the gel (usually 30-45 min). The power supply was then turned off , the gel was removed from glass plates and stained immediately.

2.2.3.5 Protein Staining and Destaining

After the electrophoresis, the gel was stained for proteins in the staining solution (solution g) overnight and then destained with first destaining solution for 1 hr and finally soaked in second destaining solution.

## 2.2.4 Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE)

### 2.2.4.1 Stock Solutions

a) 30 % Acrylamide

30 g of acrylamide was dissolved in 30 ml of distilled water , the volume was adjusted to 100 ml and stored in the dark bottle at 4 °C.

b) 1 % Bis-acrylamide

1 g of bis-acrylamide was dissolved completely in 100 ml of distilled water.

c) 50 % Sucrose

50 g of sucrose was dissolved in 50 ml of distilled water , the volume was finally adjusted to 100 ml with distilled water.

d) 0.02 M Ammonium Persulfate

0.352 g of ammonium persulfate was dissolved in 1 ml of distilled water.

e) Protein Staining Solution

The solution contained 27 % ethanol, 10 % acetic acid, 0.04 % coomassie brilliant blue R-250 and 0.5 % $CuSO_4$. (The $CuSO_4$ was dissolved in water before adding the alcohol.)

f) Protein Destaining Solution

First Destaining Solution:

The solution contained 12 % ethanol, 7 % acetic acid and 0.5 % $CuSO_4$. (The $CuSO_4$ was dissolved in water before

adding the alcohol).

Second Destaining Solution:

The solution contained 25 % ethanol and 7 % acetic acid.

2.2.4.2 Preparation of IEF-PAGE

A gel mixture containing 5.4 % acrylamide, 0.2 % bis-acrylamide, 11.86 % sucrose and 1.98 % ampholine (pH range 5-7) , 0.06 % (V/V) TEMED and 0.1 mM ammonium persulfate was performed in the space between a glass plate attached to gel support film and casting tray. The gel solution was prepared as described in the following table. It was left to polymerize overnight at room temperature.

| Solution | volume (ml) |
|---|---|
| 30 % Acrylamide | 0.9 |
| 1 % Bis-acrylamide | 1.25 |
| Ampholine pH 5-7 | 0.243 |
| Distilled water | 1.39 |
| 50 % Sucrose | 1.186 |
| 0.02 M Ammonium persulfate ($\mu$l) | 39.5 |
| Total volume | 5.0 |

Approximately, 2 $\mu$l of sample containing 5 mg/ml of protein concentration was applied to the gel.

2.2.4.3 IEF Operation

Electrofocusing was carried out horizontally under constant voltage in a stepwise increase of 100 V for 15 min , 200 V for 15 min and 450 V for 1 hr.

2.2.4.4 Staining and Destaining

Following electrophoresis, the gel attached to gel support film was soaked in the staining solution overnight. The gel was then immersed in two or three 200 ml changes of first destaining solution until the background was nearly clear. Afterwards it was transferred to the second destaining solution.

2.2.5 Determination of Proteins

2.2.5.1 Absorbance at 280 nm ($A_{280}$)

Absorbance was commonly used for generating a protein elution profile after column chromatography, using 0.01 M acetate buffer pH 4.15 for blank.

2.2.5.2 Lowry Assay

Protein concentration was determined according to Lowry method (Lowry *et al.*, 1951) with bovine serum

albumin as standard. A standard curve was prepared from BSA solution containing 10 to 100 $\mu$g protein in a final volume of 0.1 ml. Three milliliters of the solution containing 1:1:100 of 0.5% $CuSO_4.5H_2O$ : 1% sodium potassium tartrate : 2 % $Na_2CO_3$ anhydrous in 0.1 N NaOH was added to 0.1 ml protein sample, mixed and left staining for 10 min. Phenol reagent was then added and mixed. After 30 min the absorbance at 750 nm was measured comparing with standard curve of BSA.