CHAPTER II

MATERIALS AND METHODS

1. Specimens

Single voided urine specimens were collected at about 10.30 a.m. from two groups of people:

(a) one hundred and forty workers of a battery factory as exposed group

(b) one hundred and five workers of a flour factory as control group

The specimens were analysed for urinary coproporphyrin on the same day of collection. The ramaining portions of the urines were kept in refrigerator without preservative added. They were analysed for urinary delta-aminolevulinic acid and creatinine within 48 hours and for urinary lead within 7 days.

2. Determination of urinary coproporphyrin

The concentration of coproporphyrin in urine was determined according to the method of Soulsby and Smith.[76]

2.1 Reagents

- Glacial acetic acid (E. Merck Co.)
- Anesthetic ether (May & Baker Co.)
- Hydrochloric acid-iodine solution

one milliliter of 1% iodine in ethanol was mixed with 200 ml 5% hydrochloric acid before use.

2.2 Equipment

- Glass-stoppered tubes (125 x 15 mm)
- Vacuum pump (Arthur H. Thomas Co.)
- Water bath (Elconap)
- Spectrophotometer (Perkin-Elmer, model 139)

2.3 Principle

Coproporphyrin and coproporphyrinogen are extracted into ether from acidified urine. Coproporphyrinogen is oxidized to coproporphyrin by shaking the ether extract with a solution of iodine in hydrochloric acid. The solution is examined spectrophotometrically for coproprophyrin at the peak of the Soret band (maximum peak) and at wavelengths on either side (380 and 430 nm) to correct for any impurities present.

2.4 Stability of coproporphyrin in urine

Three urine specimens were determined in duplicate for CP on the day of collection and were repeated after the remaining portions were storaged 24 hours in the following conditions:

(1) at room temperature,

(2) at room temperature with 0.3% sodium carbonate,

(3) at 4°C in refrigerator.

2.5 Method

Two milliliters of a well-mixed urine were placed in a glass-stoppered tube and 0.2 ml acetic acid and 5 ml anaesthetic ether

were added. The mixture was shaken for 15 seconds and the phases were allowed to separate. The lower aqueous layer was removed. Five milliliters of hydrochloric acid-iodine solution were added. The mixture was shaken for 15 seconds and the phases were allowed to separate. The upper layer was discarded and the unstoppered tube was placed in a water bath at 37°C for 5 minutes. The tube was re-stoppered and shaken. The optical density was measured by a spectrophotometer, in a 1 cm cuvette, at wavelengths of 380, 430 nm and at the peak of the Soret band (401 nm).

The concentration of CP was calculated as micrograms per liter of urine according to the following formula:

$$\text{Coproporphyrin } (\mu g/\text{liter}) = 2D_{max} - (D_{430} + D_{380}) \times 2093 \times 1.064$$

where:

D_{max} = optical density at peak of Soret band

D_{430} = optical density at 430 nm

D_{380} = optical density at 380 nm

2093 was obtained from Rimington[64]

1.064 = correction factor

3. Determination of urinary delta-aminolevulinic acid

Urinary ALA concentration was analysed by the method of Tomokumi and Ogata.[82]

3.1 Reagents

- Acetate buffer, pH 4.6

Fifty seven milliliters of glacial acetic acid and 136 gm of sodium acetate trihydrate were added to 700 ml of

distilled water and the volume was made up to 1 liter with distilled water.

- Ethyl acetoacetate, laboratory grade (E. Merck Co.)
- Ethyl acetate, reagent grade (E. Merck Co.)
- Modified Ehrlich's reagent

Thirty milliliters of glacial acetic acid were placed into a 50-ml cylinder. One gram of p-dimethylaminobenzaldehyde, 5 ml of 60% perchloric acid and 5 ml of distilled water were added. The mixture was diluted to 50 ml with glacial acetic acid.

- Delta-aminolevulinic acid standard (Sigma Chem Co.)
- Stock ALA standard solution

Six and four-tenths milligrams of delta-aminolevulinic acid hydrochloride were dissolved and diluted with 100 ml distilled water to give a concentration of 50 mg/liter.

- ALA working standard solution

Dilutions were made from the stock solution to give concentrations ranging from 1 to 30 mg/liter.

3.2 Equipment

- Glass-stoppered tubes (125 x 15 mm)
- Vibration mixer (Lab-Line Instrument, Inc.)
- Water bath
- Centrifuge (Damon/IEC DIVISION)
- Spectrophotometer (Perkin-Elmer, model 139)

3.3 Principle

Urinary delta-aminolevulinic acid is condensed with ethyl acetoacetate to form 2-methyl-3-carbethoxy-4-(3-propionic acid) pyrrole. The pyrrole is extracted from an aqueous solution with ethyl acetate and determined colorimetrically by treating an aliquot of the extract with a modified Ehrlich's reagent.

3.4 Stability of delta-aminolevulinic acid in urine

Five urine specimens were analysed in triplicate for ALA on the day of collection and were repeated again after the urine samples were refrigerated at 4°C for 24 hours and 48 hours.

3.5 Method

One milliliter of urine was pipetted into each of two 10 ml glass-stoppered tubes and 1.0 ml of acetate buffer was added. Two-tenths of a milliliter of ethyl acetoacetate was added to one tube and the contents were mixed by a vibration mixer for about 5 seconds, the other tube was used as a blank. Both tubes were placed in a boiling water bath for 10 minutes. After cooling to room temperature, 3.0 ml of ethyl acetate were added and shaken 50 times by hand in order to extract the ALA-pyrrole. After centrifuging for 3 minutes at 1,500-2,000 xg, 2.0 ml of ethyl acetate layer were pipetted into another glass tube. Two milliliters of modified Ehrlich's reagent were added and mixed. After 10 minutes, the optical density of the color solution was determined at 553 nm against the blank.

The calculation of the concentration of urinary ALA was done by substracting the optical density of blank from that of unknown.

Then the concentration was read from a standard curve prepared by using ALA working aqueous standard solutions.

4. Determination of urinary creatinine

The determination of creatinine was based on the reaction described originally by Jaffé [42,80]

4.1 Reagents

- Picric acid solution

Twelve grams of picric acid were dissolved in 1 liter of distilled water.

- Alkaline picrate solution

Five volumes of picric acid solution were mixed with 1 volume of 10% NaOH just before use.

- Stock creatinine solution (1 mg/ml)

Creatinine zinc chloride, 0.1602 g, (Sigma Chem Co.) was dissolved in 0.1 N HCl and made up to 1 liter.

- Creatinine working standard (5 ml = 0.03 mg)

Three milliliters of creatinine stock solution were pipetted into 5 ml of 0.1 N HCl. The mixture was diluted to 500 ml with distilled water.

4.2 Instrument

- Spectrophotometer (Perkin-Elmer, model 139)

4.3 Principle

Creatinine in urine is allowed to react with alkaline picrate to form yellow-red color. The concentration is determined spectrophotometrically.

4.4 Method

One milliliter of urine sample was diluted to 100 ml with distilled water. Five milliliters of each diluted urine, distilled water and working standard solution were pipetted into each glass-stoppered tube as an unknown, blank and standard respectively. Two and a half milliliters of alkaline picrate solution were added to each tube. The mixture was shaken and allowed to stand for 20 minutes. The optical densities of standard and unknown were read at 520 nm against blank.

The following formula was used to calculate the creatinine concentration.

$$\text{Creatinine (mg\%)} = \frac{D_u}{D_s} \times 60$$

where:

D_u = optical density of unknown

D_s = optical density of standard

5. Determination of urinary lead

The urinary lead was determined according to the method of Kubasik and Volosin[49] with some modification.

5.1 Reagents

- tris(Hydroxymethyl)aminomethane buffer, 0.75 mole/l

- Calcium chloride solution, 1.04 g/liter in demineralized water

- Sodium diethyldithiocarbamate, 10 g/liter in demineralized water

- Methylisobutyl ketone (MIBK)

The solvent was equilibrated over distilled water for 24 hours before use.

- Stock lead standard, 1,000 µg/ml

- Intermediate lead standard, 10 µg/ml

- Working lead standard

Intermediate lead standard of 0.5, 1, 1.5, 2 ml were diluted with normal urine to make the final concentrations of 50, 100, 150, 200 µg/liter.

5.2 Equipment

- Vibration mixer (Lab-Line Instrument, Inc.)

- Centrifuge (Damon/IEC DIVISION)

- Atomic Absorption Spectrophotometer, Varian Techtron model AA6, with M63 carbon rod atomizer.

5.3 Principle

Lead chelated with sodium diethyldithiocarbamate is extracted at pH 7.0 into methylisobutyl ketone. The carbon rod atomizer,

in conjunction with atomic absorption spectrophotometer, is used to analyse the extracts.

5.4 Method

The instrument conditions used in this experiment were:

Wavelength	217 nm
Slit width	0.5 nm
Lead lamp current	8 mA
Carbon rod atomizer	
drying	3.5 voltage settings
	20 seconds
ashing	6 voltage settings
	15 seconds
atomizing	5.5 voltage settings
	1.5 seconds
Nitrogen flow	4 liters/minute
Fuel flow	1 liter/minute

One milliliter of each standard or urine sample was pipetted into each glass-stoppered tube. One milliliter of tris(hydroxymethyl) aminomethane buffer, 1.0 ml of the sodium diethyldithiocarbamate reagent, 0.5 ml of the $CaCl_2$ reagent, and 1.0 ml of the MIBK reagent were added to each tube. The contents of the tube were mixed on a Vortex-type mixer for 25 seconds and then centrifuged for 15 minutes at 2500 rpm. Two microliters of the ketone solution were injected into the carbon rod atomizer for analysis.

To determine the concentration of urinary lead in the unknown, the absorbances of the standards were plotted against the concentrations of the metal in the standard solutions. The standard curve was corrected for endogenous concentration of the metal by drawing a second line parallel to the first and passing through the zero origin. The concentration of unknown was read out from the second line.