CHAPTER II
MATERIALS AND METHODS

Instruments

1. Atomic absorption spectrophotometer (Shimadzu Model AA-6300, Japan) equipped with a hollow cadmium cathode lamp and autosampler (Shimadzu ASC-6100, Japan) was used for cadmium analysis.
2. Inductively coupled plasma spectrometer (Shimadzu Model ICPS-7500 sequential plasma spectrometer, Japan) equipped with autosample changer (Shimadzu AS-9, Japan) was used for calcium, phosphorus, zinc and copper analyses.
3. Water bath (INCUBATER TA 30,*Abe*, Japan) was used for β_2-MG analysis.
4. Aluminium block incubator with dry thermo unit T-482 (Taiyo Science, Japan) was used to digest samples for ICP analysis.
5. Digital diluter pipetter (Shimadzu DIP-1, Japan) was used for total amino nitrogen analysis.
6. UV-VIS spectrometer (Shimadzu Model UV-1200, Japan) with sipper (Shimadzu Sipper 160, Japan) was used for renal dysfunction markers; NAG, total protein, glucose, total amino nitrogen, β_2-MG, α_1-MG, albumin and creatinine analyses.

Chemicals

Urinary metal control samples (National Bureau of Standards) from the USA were used as a quality control material for urinary metal analysis. ICP multi-elements standard solution w-1, urinary protein and reagent kit and standard solution of Cd, Cu, Zn, Ca, and P were purchased from Wako Pure Chemical Industries, Inc. (Japan). All containers used for collecting urine samples were tested prior to use to ensure that they contained undetectable amounts of Cd. All other chemicals and reagents were specifically graded for toxic metals analysis. A list of chemicals and solution preparations is shown in Appendix B and C, respectively.

Study area

The three districts; Phrathat Phadaeng, Mae Tao and Mae Ku, in Amphur Mae Sot, Tak province, were selected as study sites. Subjects living in these 3 districts were known to have high risk of Cd exposure.

Study subjects

This study recruited 224 people, who lived in Mae Sot, had a high urinary Cd concentration and agreed to provide their blood and urine samples. They were also interviewed for information on personal and health history using questionnaire newly created for optimal information. More than 90% of the population were farmers in this area. Most of inhabitants stored and ate polluted rice, which was produced in their own paddies.

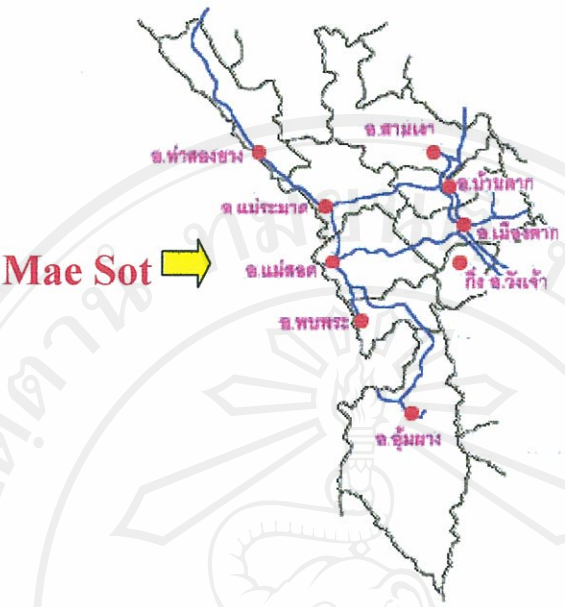

Figure 2 Location of Mae Sot (the study site) in Tak province, Thailand.

The subjects were divided into 2 groups with approximately 100 people per group. They were men and women in equal numbers.

The sample size was calculated using the following equation:

$$N = 2[(Z_\alpha + Z_\beta)(\sigma) / \mu_c - \mu_t]^2$$

where: N = sample size

Z_α=critical Z value of alpha

Z_β=critical Z value of beta

σ^2 = pooled variance

μ_c = mean of the control group

μ_t = mean of the experimental group

The assumptions were alpha of 0.05 (two-sided), which meant, $Z_\alpha = 1.96$; beta of 0.20 (one-side), which meant, $Z_\beta = 0.84$. Sample size calculation referred to the data of Cd in urine and other markers for renal dysfunction; such as NAG and α_1-MG (Nakadaira *et al*., 2003).

Therefore, $N = 2\{(1.96 + 0.84)(2.62) / 2.69 - 1.08\}^2$

$N = 41.55$

where: σ^2= pooled variance of urine Cd

μ_c = mean of urine Cd in the control group

μ_t = mean of urine Cd in the experimental group

$N = 2((1.96 + 0.84((1.86) / 6.4 - 5.1\}2$

$N = 32.1$

where: σ^2= pooled variance of NAG

μ_c = mean of NAG in the control group

μ_t = mean of NAG in the experimental group

$N = 2\{(1.96 + 0.84)(2) / 3.87 - 2.35\}^2$

$N = 27.15$

where: σ^2 = pooled variance of α_1-MG

μ_c = mean of the α_1-MG control group

μ_t = mean of the α_1-MG experimental group

More than two hundred subjects were selected from the studied areas. The subjects were divided into 2 groups according to Cd levels in their urine.

Group 1: Subjects had urinary cadmium between 5-10 μg/g Cr. This group was considered representative of highly exposed Cd people.

Group 2: Subjects had urinary cadmium over 10 μg/g Cr). This group was considered representative of very highly exposed Cd people.

Questionnaire

A newly created questionnaire was established to include informations such as age, occupational history, present health status, disease history, smoking and dietary habits, and alcohol consumption. The detail of the questionnaire is shown in Appendix D.

Sample collection

Morning urine samples were collected in polyethylene bottles after the subjects underwent physical examination including height and weight measurement. Procedures for collecting the urine were as follows:

1. Urine was collected in polyethylene bottle containers.
2. The urine samples were divided into three portions using a disposable pipette.
3. The first portion comprised about 3 ml for (Cd, Pb, Zn, Ca and P) analyses.
4. The second portion comprised about 3 ml for β_2-MG and NAG quantitation. pH of this urine portion was tested by the urine strip test. If the pH was equal to or lower than 5, one drop of 0.5N sodium hydroxide was added to adjust the pH of the urine to 6 - 8.
5. The third portion comprised about 6 ml for protein, glucose, total amino nitrogen, lysozyme, α_1-MG, Albumin and creatinine quantitation.
6. All of the urine samples were kept on ice immediately after collection.
7. All urine samples were transferred to the Mae Sot hospital laboratory and frozen at -20 ˚C until the analyses were performed.

Qualitative urinalysis

Qualitative tests of the urine for pH, protein, glucose, occult blood, urobilinogen and ketone body were determined by the urine strip test (Ames test, Bayer, Germany). The qualitative urinalysis was analysed within the day of urine collection.

Procedure of analysis

1. A fresh urine specimen was collected in a clean, dry container. A strip was briefly immersed in the urine specimen, covering all reagent areas.

2. The edge of the strip was run against the rim of the urine container to remove excess urine. The strip was held in a horizontal position.

3. The reactions were read visually. The strip test areas were compared to those on the strip color chart at the specified times. The results were recorded, and the strip was discarded.

Methodologies and interpretation

Glucose

This test was based on a double sequential enzyme reaction. One enzyme, glucose oxidase, catalyzed the formation of gluconic acid and hydrogen peroxide from the oxidation of glucose. A second enzyme, peroxidase, catalyzed the reaction of hydrogen peroxide with a potassium iodide chromogen to oxidize the chromogen to colors ranging from green to brown.

Ketone

This test was based on the development of colors ranging from buff-pink, for a negative reading, to purple when acetoacetic acid reacted with nitroprusside.

Blood

This test was based on the peroxidase-like activity of hemoglobin, which catalyzed the reaction of diisopropylbenzene dihydroperoxide and 3,3',5,5'-tetramethylbenzidine. The resulting color ranged from orange through green; and very high levels of blood might cause the color development to continue to blue.

pH

The test was based on the double indicator (methyl red/bromthymol blue) principle, which gave a broad range of colors covering the entire urinary pH range. Colors ranged from orange through yellow and green to blue.

Protein

This test was based on the protein-error-of-indicators (tetrabromphenol blue) principle. At a constant pH, the development of any green color was due to the presence of protein. Colors ranged from yellow for negative through yellow-green and green to green-blue for positive reactions.

Urobilinogen

This test was based on the modified Ehrlich reaction, in which para-diethylaminobenzaldehyde in conjunction with a color enhancer reacted with urobilinogen in a strong acid medium to produce a pink-red color.

Analysis of metals in urine samples (Cd, Zn, Cu, Ca and P)

Copper, zinc, calcium and phosphorus were analysed by inductively coupled plasma spectrometer (ICPS). Only Cd was analysed by atomic absorption spectrometer (AAS) because the diction limit of AAS for Cd analysis was better than ICPS.

Principle of AAS

AAS is a method commonly used for detection and quantitation of more than 60 metals or metalloid elements. Each element has a unique set of groups and excited states of energy. When excited, the element will absorb a particular wavelength of energy. The wavelength of the absorbed light depends on the energy levels of both the ground and excited stages of an element. The intensity of the wavelength will provide information on the quantitation of the element. In this study the concentration of Cd in urine was measured by flameless atomic absorption spectrometry.

Urine reference material No. 2670 (The National Bureau of Standards, Washington, D.C.) was used to test the accuracy and precision of the analytical method of determining Cd.

Urinary Cd concentration was corrected for the urinary creatinine concentration determined by using the reaction method of Jaffe (Bonsnes and Taussky, 1945).

Sample preparation for AAS analysis

Urine samples were mixed in a matrix modifier of palladium nitrate solution (Kanto Kagaku Reagent division, Japan) with 5% HNO_3 before being analyzed (see Appendix C). Each solution of Cd standard for the calibration curve was diluted with palladium nitrate solution to obtain 20 mg/l of palladium nitrate, and the same amount of pooled urine sample was added (matrix matched calibration method) because the slope of standard addition curves showed a difference from the slope of a simple aqueous standard. The protocol for the preparation of urine samples was as follows;

1. A 500 µl of urine sample was added into a plastic tube.
2. The 20 mg/l of palladium nitrate in 5% HNO_3 solution was added to 500 µl of urine sample in each tube and mixed.
3. The sample and standard solution were transferred to cups for AAS.
4. All of the cups were put into an autosampler and run the AAS system.

Principle of ICPS

Inductively Coupled Plasma Spectrometry (ICPS) is a very sensitive analytical technique with a high linear dynamic range (ultra-trace to main components). It is capable of analysing all elements from lithium (Li) to uranium (U) and can be applied

to solutions, solids and gasses. In ICPS-sampled material, it is transferred by an argon flow to an inductively coupled plasma in which an effective temperature of 8,000°C results in atomisation and ionisation of the material. Subsequently, the ions are extracted into a spectrometer, with which the elemental composition of the material is determined. In this study the concentration of Zn, Cu, Ca and P in urine was measured by inductively coupled plasma spectrometry.

Sample preparation for ICPS analysis

Urine samples were digested by wet ashing in $HNO_3/HClO_4$. The protocol for digestion of urine samples was as follows;

1. One ml of urine was added in the centrifugation tube.
2. One ml of conc. HNO_3 was added to each tube containing urine.
3. The tube was inserted into the hole of a box heater, and kept in 105 °C for 1 hour.
4. The 0.5 ml of conc. $HClO_4$ was added into each tube.
5. The temperature of heater was increased to 140 °C for 30 minutes.
6. One to two drops of conc. HNO_3 was added into the tubes until the color of the sample cleared.
7. The temperature of heater was increased to 160 °C, and held until the color of the samples cleared.
8. The tubes were taken out and put on a stand to cool down to room temperature.
9. One ml of 1% HNO_3 was added into each tube.
10. The tube were kept in an incubator at room temperature until analysed.

The resulting solution was diluted 101 times with 0.1% HNO_3 for determination of Ca and P levels in urine samples by ICPS, while the solution was diluted 11 times with 0.1% HNO_3 for Zn and Cu determination by the same method.

Analysis of renal dysfunction markers

N-Acetyl-β-D-glucosaminidase (NAG)

Colorimetric assay was used for the determination of NAG in urine. The principle of the colorimetric assay is hydrolyzed sodio-m-cresolsulfonphthaleinyl-N-acetyl-β-D-glucosaminidase, sodium salt by N-acetyl-β-D-glucosaminidase, with the release of m-cresolsulfonphthalein, sodium salt (m-cresol purple) in urine, which is measured photometrically at 580 nm. In this study the urinary NAG was determined by a colorimetric assay (NAG test Shionogi, Sionogi Pharmaceuticals, Japan). The reaction of N-acetyl-β-D-glucosaminidase was as follows:

sodio-m-cresolsulfonphthaleinyl-N-Acetyl-β-D-glucosaminidase

↓ NAG

m-cresolsulfonphthalein (m-cresol purple), + N-acetyl-glucosamine

Procedure of NAG analysis

1. A 50 µl urine sample was added into a plastic tube.
2. Substrate reagent was incubated at 37 °C for 30 minutes.
3. One ml of substrate reagent was added into the plastic tube that contained the urine sample.
4. The sample was incubated in a water-bath at 37 °C for 15 minutes.
5. Two ml of stop solution were added into each tube.
6. The absorbance of the sample was measured against the reagent blank by using a spectrophotometer at 580 nm.

Beta$_2$-microglobulin (β_2-MG)

Urinary β_2-MG was determined by enzyme immunoassay. The samples were incubated with a polystyrene bead coated with human β_2-MG protein polyhapten and a monoclonal anti-β_2-MG antibody covalently bound to horseradish peroxidase. During incubation, the antibody could react with either β_2-MG on the bead or β_2-MG in the sample; the calibrator or the control. After a washing step, the bound enzyme was reacted with substrate. The intensity of the resulting color was inversely proportional to the amount of β_2-MG in the specimen. Urinary β_2-MG was determined by the EIA test kit (GLAZYME β_2-microglobulin-EIA Test, Sanyo Chemical Industries, Ltd., Japan).

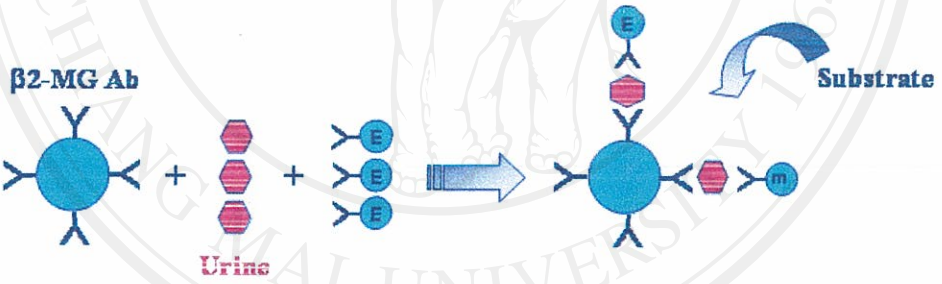

Figure 3 The reaction of β_2-MG in the enzyme immunoassay.

Procedure of β_2-MG analysis

1. The 100 µl urine sample was diluted by adding 1 ml of buffer solution.
2. A 20 µl urine sample was added into a plastic tube.
3. A 500 µl enzyme antibody reagent was added into the plastic tube that contained the urine sample.
4. One bead was added into the plastic tube.
5. The tube was incubated at 37 °C for 1 hour.
6. The solution in the sample tube was aspirated by using the aspirator tip.
7. The tube was washed 3 times by buffer.
8. The bead was transferred into another tube by using tissue paper.
9. A 500 µl color reagent was added into the plastic tube.
10. The sample was incubated in a water-bath at 37 °C for 30 minutes.
11. Three ml of stop solution were added into each tube.

12. The absorbance of the sample was measured against the reagent blank by using a spectrophotometer at 492 nm.

Alpha$_1$-microglobulin (α_1-MG)

Urinary α_1-MG was determined by immunoturbidimetric assay. Samples were incubated with a polystyrene bead coated with human α_1-MG protein polyhapten and a monoclonal anti-α_1-MG antibody covalently bound to horseradish peroxidase. During incubation, the antibody could react with either α_1-MG on the bead or α_1-MG in the sample; the calibrator or the control. After a washing step, the bound enzyme was reacted with substrate. The intensity of the resulting color was inversely proportional to the amount of α_1-MG in the specimen. Urinary α_1-MG was determined by the EIA test kit (Eiken α_1-M-III, Eiken Chemical industries, Ltd., Japan).

Procedure of α_1-MG analysis

1. A 100 µl urine sample was diluted by adding 1 ml of buffer solution.
2. A 20 µl urine sample was added into a plastic tube.
3. A 500 µl enzyme antibody reagent was added into the plastic tube that contained the urine sample.
4. One bead was added into the plastic tube.
5. The tube was incubated at 37 °C for 1 hour.
6. The solution in the sample tube was aspirated by using the aspirator tip.
7. The tube was washed 3 times by buffer.
8. The bead was transferredinto another tube by using tissue paper.
9. A 500 µl color reagent was added into the plastic tube.
10. The sample was incubated in a water-bath at 37 °C for 30 minutes.
11. Three ml of stop solution were added into each tube.
12. The absorbance of the sample was measured against the reagent blank by using a spectrophotometer at 592 nm.

Albumin

Albumin in urine samples was quantitatively determined by the immuno-nephelometry method using a kit (Micro Alb Nittohbo, Nittohbo Medical Co., Japan). The principle of the method is that when a sample is mixed with buffer and the antibody of test kit, albumin in the sample combines specifically with anti-human albumin antibodies in the reagents to yield an aggregate, which that causes increased turbidity. The degree of turbidity can be measured optically and is proportional to the amount of albumin in the sample.

Procedure of albumin analysis

1. Twenty µl of standards and urine sample were pipetted into a plastic tube.
2. A 300 µl buffer reagent was added into each tube.
3. After incubation for 5 min at 37 °C, 100 µl of albumin Ab reagent were added into each tube.

4. After incubation for 5 min at 37 °C, the absorbances at were measured 340 nm.

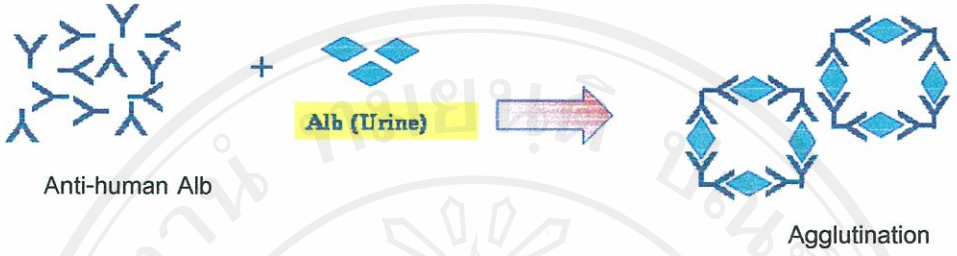

Figure 4 The reaction of albumin in the immunonephelometric method.

Total protein by the Kingsbury-Clark method

Proteins in urine samples were determined by using Sulfo-salicylic acid. The principle of this method is that when protein in urine is mixed with 3% sulfo-salicylic acid, the protein is precipitated and the degree of precipitate can be measured by a spectrometer.

Procedure of total protein analysis

1. One ml of urine was added into each of 2 tubes (a pair of tubes).
2. Three ml of 3% sulfo-salicylic acid solution were put into one of the tubes, and 3 ml of distilled water into the other.
3. After mixing, the tube were kept at room temperature for 30 minutes.
4. The absorbance was measured at 620 nm by a spectrometer.

Creatinine

Creatinine was determined by colorimetric assay (Jaffe method). The principle of this method is that creatinine forms a yellow-orange coloured compound with picric acid in alkaline solution. The concentration of the dye formed is a measure of the creatinine concentration.

Procedure of creatinine analysis

1. Five µl of urine were added into each tube.
2. Five ml of 5% picric acid with water, and 2 ml of distilled water were added into each tube containing urine.
3. The 0.5 ml NaOH solution was added into each tube.
4. After mixing, the tubes were kept at room temperature for 30 minutes.
5. The absorbance was measured at 520 nm by a spectrometer.

Lysozyme

Lysozyme (muraminidase) is an enzyme originating from phagocytic cells that catalyse the hydrolysis of the peptidoglycane layer of the bacterial cell wall. The urinary excretion of this enzyme increases during urinary tract infections, proximal tubular damage, and excessive endogenous synthesis, which exceeds the absorption capacity of the proximal tubulus. Therefore, an increase of lysozyme in urine may not be due to proximal damage, and false positive reactions may occur in urinary tract infections.

Procedure of lysozyme analysis

1. Fifty ml of 1% agarose gel mix solution was poured into a plate mold.
2. A hole was made with an aspirator.
3. Twenty µl of urine and standard solution were added into each hole.
4. The plate was kept at room temperature overnight.
5. The next morning, the diameter of each sample was measured.

Total amino nitrogen

Procedure of total amino nitrogen

1. Fifty µl of urine were added into each tube.
2. Five ml of TNBS-R were added into each tube containing urine.
3. After mixing, the tubes were kept in a dark room overnight.
4. Five 5 ml of 2N HCl was added into each tube and mixed.
5. The absorbance was measured at 330 nm by a spectrometer.

Glucose

Glucose was quantitatively determined by the Glucose C2 kit {Mutarotese-GOD method} (Wako Pure Chemical Industries, Ltd., Japan). The principle of this method is enzymatic. When a sample is mixed with a color reagent, glucose in the sample is transformed from α-form to β-form by the action of mutarotase in the color reagent. β-D-glucose is oxidized and yields hydrogen peroxide by the action of glucose oxidase (GOD). In the presence of peroxidase (POD), the hydrogen peroxidase formed yields a red pigment by quantitative oxidation condensation with phenol and 4-aminoantipyrine. The glucose concentration is obtained by measuring absorbance of the red color.

Procedure of glucose analysis

1. Three tubes were prepared for glucose analysis.
2. The tube for sample analysis had a urine sample of 0.02 ml added.
3. The tube for standard analysis had a standard solution of 0.02 ml added.
4. The tube for reagent blank analysis had either nothing or 0.02 ml of distilled water added .
5. Three ml of color reagent were added into each tube.
6. The mixture was mixed well and incubated for 5 minutes at 37 ^{O}C.
7. The absorbance (E_S) of sample (S) and the absorbance (E_{Std}) of standard (Std) were measured against the reagent blank (Bl) by using a spectrometer at 505 nm.

Data analysis

Statistical analyses were performed using the SPSS statistical package (version 12). Logarithmic transformation was applied to some of the variables to achieve a normal distribution before being subjected to data analysis, where the appropriate mean and standard deviation of urinary Cd were calculated. Simple correlations between indices of Cd exposure and renal dysfunction markers were tested by the Pearson's correlation test and/or Spearman's rho correlation before multiple regression analysis was used to compute partial correlation coefficients. One-way ANOVA was used to determine statistical significant levels among means.