4. MATERIALS AND METHODS

4.1 Equipment

Automated Chemistry analyzer,Abbott CCx (Abbott Laboratories)

4.2 Samples used in the studies

4.2.1 Urine

Normal control group : One hundred samples of second morning voided urine without preservative from 35 - 50 year-old healthy individuals, without history of diabetes mellitus or renal diseases. All specimens were confirmed for Albustix negative. They were centrifuged and the supernates were kept at -20 °C for analysis.

Diabetics group : Second morning voided urine were collected from 220 diabetics at Diabetic Control Clinic, Buddhachinnaraj hospital. Urine were centrifuged, kept sediment for urinalysis and supernate were frozen at -20 ° C until analysis within 3 days.

4.2.2 Blood

Diabetic patients were overnight-fasted and venous blood were collected in 3 tubes separately :

1. NaF tube : Plasma were separated for glucose determination.

2. EDTA tube : Hemolysate were prepared by mixing whole blood with hemolysing solution at -20 °C for HbA_{IC} determination.

3. Plain tube : Serum were separated within 1 hour for BUN, creatinine, and fructosamine determination.

4.2.3 Quality control samples

Normal level control : Pooled urine from healthy persons with normal urinalysis and Albustix negative were centrifuged and the supernatant were aliquoted in epperndorf tubes, kept at -20 °C until used.

High level control : Pooled normal urine was added with high level standard human albumin and standard NAG solutions to have albumin of 94.0 mg/dl and NAG 55.0 U/l, then aliquoted and kept at -20 °C until used.

4.2.4 Historical records of the patients

Each patient was recorded for signs and risk factors for diabetes mellitus, blood pressure, weight and disease history.

4.3 Methods

4.3.1 Microalbumin in urine (Pongsomboon S, 1992)

At pH 3.0, albumin acts as a cation to bind the anionic dye, Bromphenol blue. There is high binding affinity between the dye and albumin so that small changes in the ionic strength, pH or the presence of competing ligands will not break the dye-protein complex. The test was applied with the automated chemistry analyzer, Abbott CCx, by setting parameters as follows in appendix 8.1. The values obtained were then calculated and reported in term of mg/gm creat.

4.3.2 N-acetyl-β-D-glucosaminidase (NAG) (Makise J, *et al.*, 1988)

2-Chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG) was hydrolyzed by N-acetyl-β-D-glucosaminidase (NAG) at 37 °C pH 4.8, liberated the chromogen, CNP. NAG activity is determined by calculating the rate of CNP formation in terms of the change absorbance per minute at 400 nm. The test was applied to automated chemistry analyzer CCx by setting parameters as follows in appendix 8.2. The NAG activity were reported as U/gm creat.

4.3.3 HbA_{1C} (Klenck DC, *et. al.*, 1982)

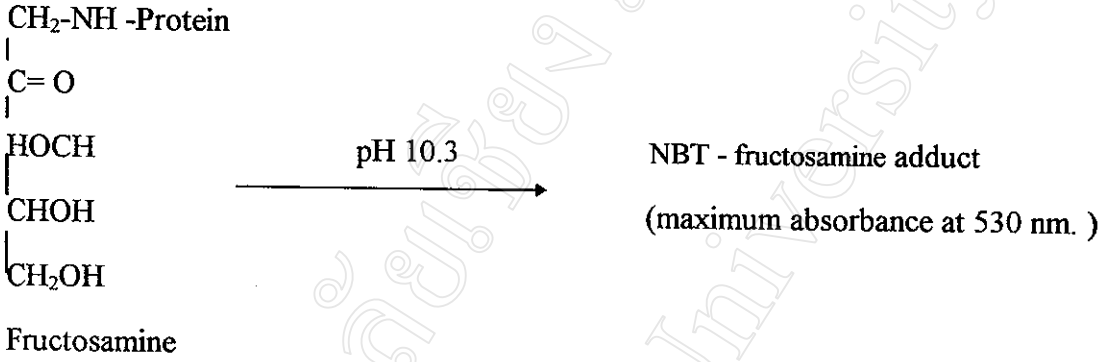
The Helena Glyco-Tek affinity column method employs an affinity column utilizing a dihydroxyboryl group bound to an insoluble cellulose resin. The unique property of the dihydroxyboryl group is the high affinity to bind with the cis-diol groups presented in many simple sugars including glucose, thus, allowing separating of glycated hemoglobins in hemolysate from non-glycated ones. Elution with a slightly basic buffer to removes non-glycated hemoglobin, carbamylated hemoglobin and the labile form of Hb_{A1}, while retaining the glycated form. The glycated hemoglobin is eluted with polyhydric alcohol, sorbitol, and determined by comparison of the two solutions utilizing a spectrophotometer operating at 415 nm.

$$\% \text{ G-Hb} = \frac{\text{Abs. G-Hb tube} * 100\%}{(\text{Abs. of G-Hb tube}) + 5.0\ (\text{Abs. of non-G-Hb tube})}$$

$$\% \text{ Hb } A_{1C} = 0.6846 * \% \text{ G-Hb} + 0.973258$$

4.3.4 Fructosamine (Baker JR, *et. al.*, 1985)

The assay was based on the reduction of nitroblue tetrazolium (NBT) salt in alkaline solution compared to 1-deoxy-1-morpholinofructose (DMF) reaction as standard.

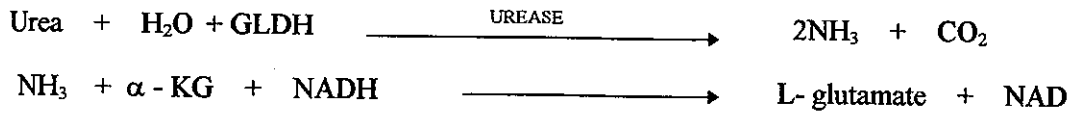


Fructosamine

The reaction was performed with automated Abbott CCx by setting parameters as in Appendix 8.3.

4.3.5 BUN

Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. In the presence of glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide (NADH), the ammonia combines with α-ketoglutarate (α-KG) to produce L-glutamate. Adenosine diphosphate (ADP) is included as an activator and stabilizer of GLDH. The reaction is monitored by measuring the rate of decrease in absorbance at 340 nm. as NADH is converted to NAD



4.3.6 Creatinine

Creatinine reacts with alkaline picrate to produce a reddish color (the Jaffe reaction). The yellow-reddish color formed is directly proportional to the creatinine concentration and is measured photometrically at 500-520 nm.

4.3.7 Glucose (Hexokinase)

Hexokinase catalyses the phosphorylation of glucose by ATP producing ADP and glucose-6-phosphate (G-6-P). G-6-P is oxidized to 6-phosphogluconate with the reduction of NAD^+ to NADH by glucose-6-phosphate dehydrogenase (G-6-PDH). The amount of NADH formed is proportional to the concentration of glucose in the sample and can be measured by the increase in absorbance at 340 nm.

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

$$\text{G-6-P} + \text{NAD}^+ \xrightarrow{\text{G-6-PDH}} \text{6-Phosphogluconate} + \text{NADH} + \text{H}^+$$

4.3.8 Urinalysis

The second morning-voided mid-stream urine of healthy and diabetics were collected and examined for physical appearance : color, specific gravity, turbidity and chemical substances, *i.e.*, ; blood, protein and glucose using Albustix. After that, the urine were centrifuged and the supernatant were collected for microalbumin and NAG assay. The precipitated were collected for microscopic examination. Criteria of urinalysis for renal disease : Proteinuria 1+ or upper, coarse granular casts or red cell casts > 1 per low power field (LPF), abnormal in urine physical and chemical examination.

4.4 Method of studies

4.4.1 Precision studies

Within-run and between-run of each test were evaluated by analyzing of one control serum or urine specimen 10 times at the same run and different day respectively. The results obtained were calculated for mean, standard deviation (SD) and % coefficient of variation (%CV).

4.4.2 Linearity studies of MA and NAG

Human serum albumin solution was prepared into various concentrations: 0, 12.5, 50 and 100 mg/dl then analyzed. The final absorbance at 630 nm. were plotted against albumin concentrations.

NAG were prepared by diluting NAG standard (6.6 unit per mg. protein, Sigma) 200 µl with distilled water to get NAG activity of 64.52 U/l then make a two fold dilution to give 32.26, 16.13 U/l and analyzed. The final absorbance at 415 nm. were plotted against NAG activity (U/l).

4.4.3 Accuracy studies

The percentage recovery of microalbumin and NAG activity were determined by using the pooled urine in which known amount of microalbumin and NAG activity were added with various volume ratio (4:1, 3:2, and 2:3). The mixture were then assayed and calculated for percent recovery of standard added. Standard human albumin (100 mg/dl) was performed in the same manner of NAG.

4.4.4 Stability test of BPB and CNP-NAG reagents

BPB reagent was prepared and used for albumin determination in 20 and 100 mg/dl standards every 10 days while keeping at 4 °C. The results were compared with day 0 for verification of reagent stability.

CNP-NAG reagent was evaluated in the same manner using urine specimen with 20 and 55 U/l NAG activity and the test was repeated every 5 days duration.

4.4.5 Clinical values of urinary MA and NAG determinations

Sensitivity, Specificity of urinary albumin and NAG tests for diabetic nephropathy were evaluated by :

Sensitivity is the probability of positivity in disease person.

Specificity is the probability of negativity in healthy person.

Positive predictive value is the probability of the disease when the test is positive.

Negative predictive value is the probability of healthy while the test is negative.

Prevalence is the ratio of population which was diseased and the total of studied population.

Criteria of diabetic nephropathy (DN):

Urinalysis: RBC or coarse granular cast > 1 /LPF

Proteinuria 1^+ - 4^+

BUN > 20 mg/dl, Creatinine > 2 mg/dl

	Disease	Normal	Total
Positive	A*	B	A+B
Negative	C	D	C+D
	A+C	B+D	A+B+C+D

* A, B, C, D are the number of person with conditions indicated

Disease = Urinalysis positive

Normal = Urinalysis negative

Sensitivity = A/(A+C)

Specificity = D/(B+D)

Positive predictive value = A/(A+B)

Negative predictive value = D/(C+D)

The predictive value was varied in differrent group of population depend on the prevalence of disease.

4.4.6 Normal reference values

Second-morning urine and fasting blood serum from 100 healthy adults aged 35-55, with no history of kidney disease or diabetes mellitus, normal blood pressure (120/80 mmHg), negative for urinalysis were determined for assigning the normal reference range of urinary NAG, MA and serum fructosamine.

The criteria for normal reference value is mean ± 3SD. We also use ROC curve to determine the cut-off level of MA and NAG/gm kreat.

4.4.7 Correlation of the tests

A. Microalbumin and NAG activity

The microalbumin value and NAG activity in urine of diabetics were plotted and calculated for correlation coefficient (r).

B. Microalbumin, NAG activity and glycemic control

Urinary microalbumin and NAG activity were plotted against HbA_{1C} in diabetic patients with and without nephropathy. HbA_{1C} and fructosamine were also tested for correlation in the same manner as in A.

C. Microalbumin, NAG and urinalysis

The microalbumin, NAG and urinalysis were studied its sensitivity and specificity by using urinalysis as standard and Chi square table for analysis.