6. DISCUSSION

The monitoring of urinary N-acetyl-β-D-glucosaminidase (EC 3.2.1.30 ; NAG) is one of the most proposing, early, non-invasive tests for renal injury. During the last decade, great efforts have been made to develop rapid, reliable and cost effective methods for NAG assay. All the methods currently used involve incubation of the enzyme with a phenolic N-acetylglucosaminide which can be divided into two groups. The first group utilized end point methods (Horak E, *et al.*, 1981, Noto A, *et al* 1983 and Yuen CT, *et al.*,1987) in which an alkaline buffer is added to stop the enzyme reaction and to develop the color of the released chromogenic phenol molecule. In contrast, the second group included several recently developed kinetic rate assay methods (Makise J, *et al* .,1988 ; Noto A, *et al.*,1988 and Yagi T, *et al.*, 1989) in which the substrate incorporates a phenolic aglycone with a low pKa value for the free phenol. When the aglycone is released at pH 4.8-6.3, a color develops, obviating the need for addition of alkaline-stopping buffer. NAG activity is detectable in the urine of healthy individuals, but is greatly increased after primary or secondary renal damage resulting from renal failure, transplant rejection, nephrotoxicity and hypertension or diabetes (Jung K, *et al.*,1982). Urinary NAG activity is an especially sensitive indicator of early kidney injury. It increases before other renal function test results become abnormal (Price RG, 1982). In spite of its clinical significance, NAG is not commonly measured in the routine clinical laboratory, mainly because of the lack of suitable methods.

The proposed kinetic method described here exploited the fact that, the pKa value for CNP liberated from CNP-NAG is 5.5 which is closed to the optimal pH for the enzymatic reaction of NAG (Makise J , *et al.*,1988). The used of CNP-NAG therefore makes it possible to perform a highly sensitive rate assay without the addition of any chromogen-containing stopping reagent and it can be performed with various types of automated analyzer. CNP-NAG is formed through β-binding of N-acetyl-β-D-glucosamine and CNP, NAG can act directly on the substrate. No additional enzyme is required because the liberated CNP can be directly measured.

Disadvantages of the present method is that the final concentration of the CNP-NAG substrate can not be increased beyond 2.0 mmol/L owing to the solubility in water is limited. Additional of a surfactant, Triton X-100 can slightly increase its solubility (Makise J, *et al.*, 1988). In addition, the substrate is unstable in solution, crystals gradually formed at 4 °C. During preparation, the solubility of CNP-NAG is increased by heating the reagent up to 60 °C and Triton X-100 is not necessary, it was noticed that using Triton X-100 will shorten the stability and cause the precipitation of the reagent (Polnamin D, 1995).

Urinary NAG is an extremely sensitive index of renal parenchymal damage, a reliable test to use in monitoring the progression of kidney diseases. Patients with normal and abnormal renal function can be discriminated by analysis of NAG activity in untimed specimen of urine because there is little diurnal fluctuation in the rate of NAG excretion (Kunin C, *et al.*,1978; Price RG, *et al.*,1979b and Wellwood J, *et al.*,1975). Variations in urine flow can reliably be compensated by relating

urine NAG activity to urine creatinine concentration. NAG activity is not increased by bacterial colonization of urine (Kunin C, *et al.*,1978) and urine NAG activity is usually normal in subjects with postural proteinuria (Sheth KJ, *et al.*,1978). Urine specimens can be stored for several days at 4-6 °C and for months at -20°C without change in NAG activity. (Hultberg B, *et al.*, 1980 ; Lockwood TD, *et al.*, and Knoll E, *et al.*, 1980).

Proteinuria in renal disease may result from glomerular and/or tubular function. Tubular cell destruction causes the increased excretion of cellular enzymes into urine while increased glomerular permeability increases the urinary excretion of plasma proteins such as albumin, transferrin and the acute phase reactants, *e.g.*, alpha 1-antitrypsin and beta-acid glycoprotein (Killingsworth LM, 1982).

Many laboratories still use reagent strips for semiquantitative determinations of urinary albumin and results are graded as trace and 1+ through 4+ (1+,2+,3+, 4+ is equivalent to 0.3, 1.0, 3.0 and 20.0 or more gm/l, respectively). This procedure does not permit one to monitor small changes in albumin concentration. With the BPB method presented here, we can evaluate these small changes which is of practical value because it allows rapid and direct quantification of urinary albumin over a wide concentration range. Dipstick procedures are sensitive to changes in pH, producing erroneously high results with strongly alkaline urine (Gyre WL, 1977). Disadvantages of reagent strip is that the positive test reflexes more than 50% of renal damage and is not reversible, the detection of microalbumin in urine will predict early stage of the disease that can be treated. Urinary pH below 10.6 showed almost no interference in the described method because of the highly buffered BPB working solution

used. Dipstick methods for urinary albumin are significantly affected by salt, uric acid, creatinine, calcium and bilirubin. At great excess concentrations of these substances in urine did not significant affect albumin estimation measured by the proposed method (Shosinsky KH, *et al.*,1987). Pongsomboon S, 1992, used BPB reagent for determination of microalbumin which could not be detected by Albustix. The BPB method showed suitable precision range 3.3-4.8 % and 7.4-9.5 % for within-run and between-run, respectively. The color production completed within 1 minute and stable for at least 30 minutes. Chanarat N, *et al.*, 1990 also reported that the precision of determination of MA by bromphenol blue (BPB) reaction was between 2.0-3.0 % and 5.5-8.7 % for optimal and routine condition variances, respectively. Linearity of the reaction was at least 100 mg/dl and sensitivity was at least 1 mg/dl. Accuracy of the method by average % recovery of standard was 102.3 %.

Albumin in urine is stable and the samples can be stored at 4-20 °C for 10 days without preservation. Five freeze-thaw cycles showed no change in albumin concentration compared with the original values in fresh samples (Townsend JC, 1986). Centrifuged samples stored for 1 week at 4 °C or 1 month at -20 °C showed no difference in concentration even when visible precipitates were present (Silver AC, *et al.*, 1987).

The level of the NAG-A fraction (glomerular fraction which can be inactivated after 60 °C for 10 minutes) correlates well with the levels of urinary protein in patients with diabetic nephropathy. It was suggested that the levels of the NAG-A fraction in urine samples might be a better reflection of glomerular damage than total urinary NAG activity in patients with NIDDM (Saitoh A, *et al.* , 1992).

Microalbuminuria is assumed to be a predictor of clinical nephropathy in type I diabetes mellitus, (Viberti GC, *et al.*, 1982; Mogensen CE, *et al.*, 1984) however, microalbuminuria only indicates a glomerular involvement and does not provide information about the renal tubular function, which may also be impaired in diabetes mellitus. The measurement of NAG, a lysosomal enzyme localized in the proximal tubular cells, serves as an additional parameter for the assessment of tubular damage (Whiting PH, *et al.*,1979a). In this study, MA alone has sensitivity 94.1%, specificity 93.1%, false positive 6.4% and false negative 0.5% for detecting early diabetic nephropathy, NAG activity alone has sensitivity 75.0%, specificity 90.7%, false positive 8.6% and false negative 1.8%, combination of the two tests raise the sensitivity and specificity to 100 and 98.3%, while decrease the false positive and false negative to 1.4 and 0.0%, respectively. We also found that at different prevalences, 5.0, 10.0,15.0%, the positive predictive values were 72.5, 87.5 and 89.8%, respectively. The negative predictive values were 100.0% at 5, 10 and 15 % prevalences as shown in Table 8. Powell SC, *et al.*, 1983 found that urinary NAG did not only increase in primary but also in secondary renal disorders, such as light chain disease and myoglobinuria. The increase of NAG in urine is not entirely specific for renal damage, but also observed in other disorders such as pancreatitis, however, clinical appearance are distinct from renal damage. A great number of patients with ovarian cancer have increased NAG, probably because of the nephrotoxicity of the therapeutic drugs used.

The association of urinary NAG and MA excretion in diabetics with the degree of glycemic control, assessed in the current study

by measurement of serum fructosamine and HbA_{1C} levels. We have observed a statistically significant correlation of serum fructosamine concentration with HbA_{1C} ($r= 0.700$, $p<0.05$) means that measurement of serum fructosamine would give similar information as an HbA_{1C} estimation reflecting integration of blood glucose values over different time intervals : two weeks for fructosamine and up to eight weeks for glycated hemoglobin (Baker JR, *et al.*, 1985). Although some physicians may prefer the longer integration of blood glucose concentrations offered by measurement of HbA_{1C}. We found that measuring fructosamine has some advantages, besides the lower cost, the assay being technically simple, easily automated application and gave a precise and reliable quantification of fructosamine concentration in serum.

We find correlation between a glycemic control (HbA_{1C}) as well as microalbuminuria ($r=0.49$ $p<0.05$) in diabetes developing nephropathy. It recognized that hyperglycemia observed in both type I, II diabetes is most likely the major cause of micro- and macrovascular diseases. For glycemic control, the short-term objective in diabetes is to maintain a normal glucose level, while the long term objective is to prevent or delay the onset of micro- and macrovascular complications. In diabetics without diabetic nephropathy, the correlation coefficient (r) of NAG excretion, microalbuminuria to glycemic control (HbA_{1C}) is least, $r = 0.14$ and 0.39, respectively.

It should be noted that when glycated protein are already present, improvement of glycemic control alone may not be sufficient to prevent the continued progression of pathologic processes, such as formation of AGEs from glycated proteins and subsequent tissue damages.

In this case, pharmacologic agents, such as aminoguanidine, should be used in conjunction with glycemic control to interfere directly with the self-perpetuating process of AGEs formation and the accumulation of additional glycation process. (Wu JT, 1993)

In diabetics without nephropathy, the correlation coefficient of NAG excretion and MA is 0.48 ($n=200$ $p<0.05$) while in patients developed diabetic nephropathy, it increases to be 0.78. Glomerular changes occur early in diabetes while tubular cells can reabsorb the increased albumin loaded, resulting in normal albumin excretion rate but increased lysosomal activity and NAG excretion. The next stage is characterized by an increase in both NAG and albumin indicates that proximal cells reabsorption capacity has been exceeded. Further increase in NAG excretion will accompany with loss in the functional capacity of the cell loading to structural breakdown which ultimately results in cell necrosis .

We propose that all assays : MA, NAG activity and glycemic control have good analytical characteristics, sensitivity and specificity suitable for the assessment in clinical laboratories and suggest that the quantitation of both glomerular and tubular proteinuria provides a sensitive and cost effective instrument for the non-invasive screening for renal involvement in patients with diabetic nephropathy.