1. INTRODUCTION

Diabetes mellitus (DM) is a genetically heterogenous group of disorders manifested ultimately by insulin deficiency and /or loss of carbohydrate tolerance.The disease may be idiopathic or secondary to such disorders as pancreatitis, pancreatic carcinoma, hemochromatosis, Cushing's syndrome, acromegaly, *etc*. In 1979, the National Diabetes Data Group of the National Institute of Health (NIH) classified diabetes mellitus into 2 major types and the other types include glucose intolerance due to various causes or underlying diseases (Kumar P, *et al*., 1994).

Type I Diabetes or insulin-dependent diabetes mellitus (IDDM); this type of diabetes is caused by insufficient insulin secretion (Insulinopenia). Insulin injections are necessary to maintain normal glucose metabolism. There are evidences supported that specific human leukocyte antigens (HLA) may have a role in DM expression. HLAs are dimeric proteins produced by the major histocompatibility complexes on chromosome 6. Class II HLA including DP, DQ and DR HLA-DR3, DR4 are susceptible to 90% of type I diabetes with only minor involvement in type II. It was also reported that type I diabetics resisted to HLA- DR2. The etiology could also be from autoantibodies to islets cells, glutamic acid dehydrogenase, tyrosine phosphatase or insulin causing degeneration of beta cells.

Type II Diabetes, non-insulin-dependent diabetes mellitus (NIDDM) has no correlation between severity of the disease and blood insulin level. The onset of disease is usually after 40 years. The patients are usually obesed, require no insulin and are less prone to ketoacidosis.

The other types of DM are gestational DM and DM secondary to various underlying diseases, for examples, pancreatitis, hormonal disorders, drugs- or chemical-induced, abnormal insulin receptor and certain genetic syndromes, *etc*.

Blood glucose concentration is normally tightly regulated by the coordinated action of insulin and counter regulatory hormones. The balance is maintained between glucose production by the liver and glucose clearance to peripheral tissue, primarily muscles. Insulin released from beta cells of the pancreatic islet is constantly adjusted so that normoglycemia is maintained. Accurate assessment of chronic glucose control is one of the major difficulties in managing diabetic patients. Even patients with mild disease may show large fluctuations in blood glucose and a single glucose determination may correlate poorly with mean blood glucose levels. Measurement of glycated hemoglobin, for example, HbA_{1C} and / or glycated protein are accepted as a good assessment of diabetic control. Fructosamine is the trivial name of 1-amino-1-deoxy-fructose, a ketoamine derivative of the non-enzymatic reaction product of free sugar (usually glucose) and protein. The mode of fructosamine formation is known as 'Glycation' while blood concentration depends on the glucose level and half life of the proteins. Serum fructosamine concentration is a measurement of total glycated serum protein. Albumin is the most abundant protein, therefore, measurement of fructosamine is largely determination of glycated albumin. The half life of albumin is about 2-3 weeks, thus, fructosamine reflects the integrated determines short time control of glycemia over 2-3 weeks prior to its estimation or determines short time control of glycemia in diabetic patients. For longer time control, glycated hemoglobin is used for monitoring 6-8 weeks glycemic control.

However, the test should be selected depend on the purpose of management and severity of the disease, the shorter time control will respond more quickly to change in therapy, while the longer control should be benefit with well control patients.

Hyperglycemia is not the general cause of death in the patients. After a period of diabetics, the patients usually develop complications especially in those the blood glucose level can not be well controlled and will be the cause of death if they were not corrected on time. The complications in diabetic patients can be divided in 2 groups (Kaplan A, *et al.*,1983)

1. Short-term complications. The complications that occur immediately or acutely including diabetic ketoacidosis (DKA), Non-ketotic hyperglycemic hyperosmotic coma (NHHC), infection, hypoglycemia, *etc.*

2. Long-term complications. The complications gradually developed.They usually presented after at least 5 years of diseases, those are :

2.1 Retinopathy. The principal retinopathy of diabetes is the opacity of the eye lens or cataracts. Retinopathy is also caused by proliferation of small blood vessels in the lens and the glycation of the lens' protein causing visual abnormality to blindness.

2.2 Neuropathy. Neuropathy is the most common compli-cation of diabetes mellitus. It is apparent in about 25% of diabetics and is recognized by a variety of burning sensations in extremities, dizziness and double vision. These symptoms are caused by accumulation of sorbitol, a reducing product of glucose, around nerve

ending causing water retention and decreased motor and sensory nerve conduction velocities due to axonal degeneration and demyelination.

Secondary manifestation of neuropathies include cardiac failure, excessive sweating and male impotence.

2.3 Angiopathy and coronary heart disease (CHD). Angiopathy refers to damage of blood vessel linings which increases the risk of coronary heart disease and stroke and can also leads to retinopathy and nephropathy. In diabetics, not only carbohydrate metabolism is abnormal but also protein and lipid metabolisms. Most patients have hyperlipidemia, hypertension with coincidence of platelet hyperaggregation making them easily develop coronary heart diseases.

2.4 Infections. Diabetics are highly susceptible to infections, ulceration and gangrene. Tuberculosis and skin disorders are also common in diabetics.

2.5 Diabetic nephropathy. Renal disease in diabetics is characterized by early appearance of hypertrophy of both glomerular and tubular elements, the subsequent development of thickened glomerular and tubular basement membranes associated with enhanced glomerular permeability to albumin. Diabetic nephropathy does not develop in all diabetic patients. The cumulative incidence of nephropathy is 30% to 50% in insulin-dependent diabetes (IDDM) and 10-15% in non-insulin dependent diabetics (NIDDM) which indicates that only a subset of

patients with IDDM are susceptible to the development of nephropathy. The known risk factors for the development of diabetic nephropathy are :

2.5.1 Duration of diabetes. Nephropathy is rare during the first 5 years of diabetes, after which the incidence increases until it reaches the peak at approximately 14 to 16 years of diabetes duration.

2.5.2 Systemic hypertension. Arterial hypertension regularly accompanies with establishing diabetic renal disease, exacerbates its progression, and may determine its occurrence. The predisposition to hypertension, as determined by a family history of essential hypertension and/or elevation in erythrocyte sodium-lithium counter-transport activity, may be risk factors for the development of nephropathy. This countertransport activity is believed to represent the physiologic sodium-hydrogen antiporter, which in its various forms is involved with renal sodium reabsorption, regulation of cellular pH and response to mitogens.

2.5.3 Renal hemodynamics. An elevated GFR is seen in approximately one-third of patients with IDDM and a significant proportion of patients with NIDDM. Hyperglycemia may be partly responsible for the hyperfiltration. Many factors implicated as mediators for the increased GFR present in early diabetes include growth hormone, glucagon, insulin-like growth factor type 1 (IGF-1), ketones bodies, increased dietary protein intake and altered levels or vascular responsiveness to several vasoactive peptides including cathecholamines, atrial natriuretic peptide, prostaglandins, the renin-angiotensin system and nitric oxide. Glomerular hypertension may occur independently of systemic hypertension and may be an important pathogenic factor in the initiation and progression of diabetic nephropathy.

2.5.4 Glycemic control. The effects of hyperglycemia on the development of nephropathy may be mediated by several pathogenic mechanisms. The first is the accumulation of advanced glycosylation end products. Glucose reacts with tissue protein to form intermediate Amadori products, a covalent non-enzymatic reaction products which the rate of formation is proportional to the glucose concentration. Amadori products are slowly converted into advanced glycosylation end products. The functional consequences of this formation include increased cross-linking of structural proteins, dysregulation of enzyme systems and abnormalities in the ability of certain proteins to bind regulatory molecules, for example, receptors on macrophage which in turn will stimulate the cytokine system. The second potential mechanism by which glycemic control could contribute to nephropathies by glycosylation of circulating proteins particularly albumin induces the mesangium proliferation and increase resulting in their hyperfiltrable through the glomeruli. Another mechanism is a consequence of sorbitol accumulation causing the decrease in reduced nicotinamide-adenine dinucleotide phosphate (NADPH), glutathione and myoinositol which affect on various pathways of metabolism. The accumulation of sorbitol has been also implicated in diabetic retinopathy and nephropathy.

2.5.5 Genetic predisposition. There are four factors providing evidence that there is a genetic susceptibility for the development of diabetic nephropathy including familial clustering of nephropathy, the association between nephropathy and a family history of hypertension, the similarity of renal lesions in diabetic siblings and familial clustering of nephropathy in multiplex diabetic families. Once diabetic nephropathy established, progression to end stage renal failure (ESRD)

inevitably occurs. Therefore attention has focused on intervening at as early stage as possible. Factors that portend a high risk regarding the cause of diabetic nephropathy such as albumin excretion rate, will identify a target population to direct early therapeutic intervention in an attempt to prevent or delay overt disease.

In Thailand, major complications recorded at various hospitals include hypertension, cardiovascular diseases, retinopathies and renal diseases. Minor complications found are infections, peripheral neuropathies, hypo- or hyperglycemia and coma. Prevalence of renal complication at Siriraj, Chulalongkorn, Rajvithi, Srinakarin and Lerdsin hospitals were 8.6, 17.1, 11.2, 12.8 and 16.7 %, respectively.(Tanami J, 1995).

In this thesis, we focused on diabetic nephropathy which is usually occured after about 5-10 years of diabetes and quite high in Thai diabetics. The purpose is to verify methods for detection of the disease as early as possible because it was realized that well controlled both of blood glucose level and also blood pressure is suggestive to reduce or delay the onset of renal diseases.

The hallmark of overt diabetic nephropathy is 'Albustix' positive proteinuria (Clinical proteinuria) which is invariably associated with long duration of diabetes, systemic hypertension, elevated serum creatinine and retinopathy. The earliest functional abnormality in the diabetic kidney disease is renal hypertrophy associated with a raised glomerular filtration rate which appears soon after diagnosis and is related to poor glycemic control. The initial structural lesion in the glomerulus is thickening of the basement membrane. Associated changes may result in disruption of the protein cross linkages that

make the membrane an effective filter. In consequence, a progressive leak of protein into the urine occurs. The earliest evidence of this is 'microalbuminuria' which in turn, after some years, progress to intermittent albuminuria followed by persistent proteinuria. (Rowe DJF, *et al.* , 1990)

'Microalbuminuria' has been defined as an increased excretion of albumin above the reference range for healthy non-diabetic subjects but is undetectable by the Albustix test. Urinary albumin excretion rate persists between 20 and 200 μg/min in an overnight or 24-hour sample on at least 2 of 3 occasions within a period of 6 months. An early detection of microalbuminuria is essentially attempted to determine in routine laboratory for preventing nephropathy and monitoring diabetes management. (Morganson CE, *et al.*, 1986). Various techniques have been used for measuring microalbumin in urine:

1. Radial immunodiffusion. (Mancini G, *et al.*,1965)The diffusion of albumin in starch medium containing anti-albumin will react together and form the precipitated ring which the diameter varies directly with albumin concentration.

2. Immunoturbidimetry. (Harmoinen A, *et al.*, 1969) Anti-human albumin reacts with urinary albumin forming turbidity which can be measured at 340 nm. The turbidity of the complex varies with microalbumin concentrations.

3. Radioimmunoassays. (Woo J, *et al.*,1978) This involves the double antibody technique by incubation of diluted urine at room temperature with I^{125}-labelled albumin and a rabbit antiserum monospecific

for human albumin. The main disadvantages of this method are isotope-related health, safety hazards and the short shelf-life of reagents.

4. Enzyme immunoassays. (Fielding BA, *et al*,1983; Silver AC, *et al.*,1986; Chesham J, *et al.*, 1986). The solid phase-binding double antibody assay by sensitizing the microplate wells with rabbit anti-human albumin as the first antibody and using peroxidase labelled goat anti-human albumin as the second antibody.

5. Latex agglutination. (Paoli C, *et al.*,1987) The suspension of polystyrene latex particles coated with anti-human albumin is added to the test sample. Agglutination will occur if there is albumin in the sample. The agglutination will be graded as 1+ to 4+ depend on the albumin concentrations.

6. Dye binding methods. (Schosinsky KH, *et al.*,1987 and Pongsomboon S, 1992) The procedure is based on the properties of albumin to bind with various dyes at appropriated pH, for example, binding with bromphenol blue at pH 3.0 and absorption of the BPB-albumin complex is determined spectrometrically at 630 nm.

The modification of dye binding method to be used as dipstick. The example of this method is using the dye, tetrabromphenol blue, is buffered at pH 3, it is yellow ; the addition of increasing concentrations of protein or albumin changes the color to green and then to blue. The developed color is compared with a color chart which enables the protein concentrations to be graded. The positive result with the dipstick method will be occured only with urine containing 0.3 gm/l or higher so microalbiminuria can not be detected.

Tubular proteinuria may occur as a result of a tubular defect in the handling of proteins. In tubular proteinuria, less than 2 gm./day

of proteins excreted, mostly molecular weight are less than 50,000 Da, including enzymes : amylase isozyme, ribonuclease, N-acetyl-glucosaminidase and alanine aminopeptidase, polypeptides hormone (calcitonin, follitropin, lutropin, *etc.*) and other proteins (retinol-binding protein, α_2-microglobulin, β_2-microglobulin).

Urinary tract infection (UTI) may occur in the bladder (cystitis) or involve the kidneys (pyelonephritis). The presence of urine bacterial contamination of more than 100,000 colonies /ml. is diagnostic of urinary tract infections. Urine from the patient will have an increased number of white blood cells. The presence of white blood cell casts indicates pyelonephritis. An increased number of red blood cells may also be present in the urine. There are reports that many enzymes usually of tubular origin (NAG, Aspartate transaminase, Alanine transaminase, *etc.*) increase in urine of diabetics.

N-acetyl-β-D-glucosaminidase (EC 3.2.1.30; NAG) is responsible for the degradation of mucopolysaccharides and glycoproteins. Urinary NAG has its origin largely in the epithelial cells of the proximal tubule which contain a particularly large number of lysosomes (Shrivastava KS, *et al.*,1974). Due to high molecular weight, NAG in serum can not penetrate into the glomerular filtrate if the glomerular membrane is intact. Under normal circumstance the low level of NAG in the urine represents leakage as part of exocytosis and pinocytotic activity of the tubular epithelial cells. An elevated urinary NAG occurs as a result of increased metabolic activity due to seriously increased glomerular filtration and the associated decrease in reabsorption. Increased excretion of exogenous substances

many of which are attacked by lysosomes or destruction of cells in the proximal tubule.

An increase in NAG excretion in urine must invariably be interpreted as a sign of proximal cell dysfunction. (Price RG, 1979a). The first occurrence of elevated NAG activity is usually a sign of reversible process. It has been proposed that urinary NAG assays might have considerable potential for investigating and detecting early diabetic nephropathy (Whiting PH, *et al.*, 1979a). Several trials investigating patients with type I and type II diabetes mellitus have noted that the NAG activity was raised in urine, and some evidences suggested that this increase occurred prior to microalbuminuria. (Martin D, *et al.*, 1990).

During the last decade, great efforts have been made to develop rapid, reliable and cost effective methods for NAG assay. All of the methods currently used involve incubation of the enzyme with chromogenic, fluorogenic substrates which the product formed can be measured as follows :

1. Spectrofluorometric method. (Powell SC, *et al.*, 1983) The kinetic fluorometric assay for NAG was done in a centrifugal analyzer. NAG reacts with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide and release fluorescent derivative, 4-methylumbelliferone (7-hydroxy-4-methylcoumarin) which can be monitored at 360 nm. excitation and 440 nm. emission. This method requires special equipment, photofluorometer and gel filtration to eliminate interfering substances.

2. Spectrophotometric method.

NAG will cleave the substrate giving colored product. Many substrate can be used as follows :

2.1 p-Nitrophenyl-N-acetyl-β-D-glucosaminide (PNP-NAG) (Horak E, *et al.*,1981). The substrate dissolved in sodium citrate buffer pH 4.4 at 37 °C, enzymatic hydrolysis of the substrate liberated p-nitrophenylate ion. The reaction is stopped by adding 2-amino-2-methyl-1-propanol (AMP) buffer pH 10.25. The reaction product is measured spectrophotometrically at 405 nm. Disadvantages of this method is the requirement of a sample blank for elimination of interference from many substances, *i.e.*, hemoglobin, methylene blue and salicylic acid.

2.2 m-Cresolsulfonpthaleinyl-N-acetyl-glucosaminide (MCP-NAG) method (Noto A, *et al.*,1983). Urinary NAG reacts with MCP-NAG as substrate at 37 °C for 15 minutes. The reaction is stopped by adding 0.3 mol/l Na_2CO_3. The reaction product is measured spectrophotometrically at 580 nm. This method requires no sample blank but is a two-step equilibrium assay, in which a stopping reagent is added to induce color generation in the alkaline medium. It is therefore time-consuming and requires a particular type of automated analytical apparatus. Another disadvantages is that the results are affected by drugs such as phenol or bromsulfonphtalein which are visibly colored in alkaline solution and therefore cause misleading results.

2.3 3-Ammonium-5-[4-(2-acetamide-2-deoxy-β-D-gluco-pyranosyloxy)-3-methoxyphenylmethylene]-2-thioxythiazolidin-4 one-3-ethanoate (VRA-GlcNAc) as a chromogenic substrate for NAG (Pocsi I, *et al.*, 1992). The phenol released by enzyme action has an intense absorption peak at 492 nm. The substrate solution may be stored at 4-7 °C for one week without any increase in the substrate blank. The sensitivity of the assay with VRA-GlcNAc as substrate is twice the assay with MNP-

GlcNAc. This is also manufactured as NAG test kit by Ames (Sanitoh A, *et al.*, 1992)

3. Kinetic colorimetric method (Makise J, *et al.*, 1988)

Kinetic rate assay of urinary NAG with 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG) as substrate. NAG hydrolyzes and liberates the chromogen, CNP and N-acetyl-D-glucose at 37 °C pH 4.8. NAG activity is determined by calculating the rate of CNP formation in terms of the change absorbance per minute at 404 nm.

4. Enzyme -linked immunoassay (Itoh Y, *et al.*, 1994)

Isozyme NAG-B is measured by using double antibody sandwich ELISA in which monoclonal antibody for purified NAG-B (from human placenta) is used as the capture antibody and monoclonal antibody for purified NAG-B conjugated with peroxidase as the second antibody. The chromogenic substrate, 2-2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), ABTS is oxidized with hydrogen peroxide formed in the reaction and the absorbance is measured at 415 nm.

Determination of microalbumin in urine is assumed to be a predictor of clinical nephropathy. 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. In order to establish a sensitive program for the clinical monitoring of renal involvement in diabetics, thereby, combining the advantages of quantitative analysis of urinary microalbumin and also the measurement of NAG, a lysosomal enzyme localized in the

proximal tubules, should be served as parameters for the assessment of both glomerular and tubular damages.

For microalbumin determination we developed a procedure based on the dye binding property of microalbumin with bromphenol blue (BPB). The determination of enzymatic activity of NAG, we use a new synthetic substrate, 2-chloro-4-nitrophenyl-N-acetyl-β-D-glucosaminide (CNP-NAG) as a single kinetic substrate reagent, both methods were applied to be used with the automated chemistry analyzer (Abbott Spectrum CCx). The investigations were made for studying the relationships of microalbumin in urine, NAG activity, serum urea nitrogen (BUN), serum and urinary creatinine, glycemic control parameters (Fasting blood sugar, HbA_{1C} and fructosamine), urinalysis and also the patient's history of being diabetes.