MEDICAL CHANNEL

Original Article

ESTIMATION OF URINARY ALBUMIN IN DIABETIC PATIENTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Background: Albumin appears in urine in two forms, immunoreactive and immuno-unreactive. Albuminuria estimation by immunoassay techniques detected only immunoreactive albumin in the urine of diabetic patients.
Methods: High performance liquid chromatography (HPLC) was used to measure albuminuria in twenty four hours collected sample of urine from patients with diabetes and from thirty healthy non-diabetic volunteers. Aliquots of urine samples were stored at –20°C until testing. Before analyzing for albumin by HPLC, the samples were thawed and gently mixed by vortexing. Urinary albumin was analyzed by using a reversed-phase C5 column with ultraviolet detection at 280 nm. The sample solution injected into the HPLC system and albumin elutes as a sharp peak at approximately 2.8 min with an overall runtime of 8 min.
The concentration of albumin in urine was determined by comparing the peak areas of albumin in test (unknown concentration) with peak areas of albumin in known concentration standard solutions by using formula. Six ml of blood was also collected from all study participants to estimate fasting plasma glucose, serum albumin, serum creatinine and blood urea level. Urinary creatinine was also determined and creatinine clearance was estimated by Cockcroft-Gault formula.
Results: Patients with type I diabetes were younger ($p<0.05$) while patients with type 2 diabetes were obese ($p<0.01$). Significant difference in mean duration of diabetes was found between patients with type I and type II diabetes ($p<0.05$). Patients with diabetes (type I and II) had significantly increased mean fasting plasma glucose and significantly lower serum albumin level ($p<0.01$). A non-significant difference was observed in blood urea, serum creatinine, urinary creatinine and in creatinine clearance ($p>0.05$) while excretion of mean urinary albumin was significantly increased in diabetic patients (type I & II) compared to control subjects ($p<0.01$)
Conclusion: Study showed that to determine albuminuria accurately, there is a need to assess urinary albumin by HPLC.

Key Words: Diabetes, Microalbuminuria, High Performance Liquid Chromatography.

INTRODUCTION:

Albumin belongs to the class of simple proteins that is soluble in water and coaguable on heating [1]. It is also soluble in moderate concentration in salt solution[2]. Found abundantly in human blood plasma albumin is synthesized exclusively by the liver [3]. Albumin constitutes 60% of total plasma protein and is responsible for nearly 70% of the colloidal osmotic pressure [4]. Albumin performs several important functions in human. It binds with a variety of ligands such as free fatty acids, metal ions, drugs and metabolites [5]. Albumin plays an important role in drug delivery, efficacy and detoxification[6].

Besides this albumin exerts oncotic pressure that holds water in the vascular compartment[7]. Albumin contains free cysteine residue which is the major extracellular source of thiols [8]. Albumin acts as scavenger of reactive oxygen and nitrogen species [9] .

Albumin is excreted in the urine in very small amounts of less than 30 mg/day under physiological conditions [10] . Persistent albuminuria in the range of 30-300mg/day is recognized as one of the earliest indicators of nephropathy in patients with type 1 and type 2 diabetes mellitus and a marker of progressive kidney disease [11]. Moreover, it has been recognized as a powerful marker and a predictor for cardiovascular disease and overall mortality in diabetes and in general population as well [12] .

Since chronic renal failure and cardiovascular disease are the leading causes of death in diabetics therefore measurement of albuminuria is of great importance to be able to identify those at risk in order to be able to start treatment[13].

Therefore the aim of the present study is to measure the urinary albumin in a 24 hours collected samples of urine of both type 1 and type 2 diabetic patients by high performance liquid chromatography and to compare it with the urinary excretion of control subjects to find out the difference in urinary albumin excretion rate.

METHODOLOGY:

This cross sectional observational study was carried out in the department of Biochemistry, Basic Medical Sciences Institute, Jinnah Postgraduate Medical Centre, Karachi in collaboration with the diabetic clinic of JPMC Karachi.

A total of 90 subjects of both sexes according to selection criteria were included in the study. Among them thirty diagnosed type I and thirty diagnosed type II diabetic patients were selected from the diabetic clinic of JPMC Karachi. None of the selected diabetic patients had prior history of renal diseases, hypertension, cardiac or pulmonary disease, use of any anti-hypertensive or cardiac medications and tobacco use. Patients with chronic renal failure or on dialysis or giving histories of thyroid and /or growth hormone abnormalities were excluded from the study. Similarly, pregnant women and patients doing physical exercises were also not included in the study. All selected diabetic patients had negative urinary dipstick test. While thirty sex matched normal healthy subjects from the general population were taken as control. Healthy volunteers had fasting plasma glucose level de 126 mg/dl and had negative urinary dipstick test.

At the time of recruitment all the participants were explained and provided a detail information leaflet about the study objectives, procedures and the risk involved. The participants were reassured about the confidentiality of data. An informed consent was signed by each participant.

An interviewer administered questionnaire including demographic, anthropometric and clinical parameters including family and personal medical history was collected from each consented participants.

Anthropometric measurements including weight and height were obtained using standardized techniques. Folding screens were used to isolate the subjects. Blood pressure was recorded in the sitting position in the right arm to the nearest 2 mmHg with a mercury sphygmomanometer.

ESTIMATION OF URINARY ALBUMIN:

Urine collection (24 hour collection):

Due to diurnal variation in the excretion of albumin 24 hours of urine was collected. Each participant was instructed in order to obtain proper collection of a 24-hour urine specimen.

Determination of Urinary Albumin by HPLC Method:

TABLE 1:
Comparison of biophysical biochemical variables in Type I and Type II diabetic patients with control subjects

S. No.	Parameters	Normal subjects (n = 30) M/F=17/13	Type I diabetic patients (n = 30) M/F=16/14	Type II diabetic patients (n = 30) M/F=18/12
1.	Age (Years)	51.7 ± 1.70	13.9 ± 1.04 **	50.5 ± 1.57 NS Δ Δ
2.	Height (Cm)	167.2 ± 0.85	152.40 ± 0.96**	168.67 ± 1.31 NS Δ Δ
3.	Weight (Kg)	68.33 ± 1.55	43.03 ± 1.74**	76.6 ± 1.16** Δ Δ
4.	BMI (Kg/m^2)	22.17 ± 0.45	18.6 ± 0.50**	26.83 ± 0.39** Δ
5.	Duration of diabetes (years)	0.00	8.6±0.80	5.1±0.65 Δ
6.	Serum Albumin (g/dl)	4.53 ± 0.12	3.12 ±0.15**	3.16±0.14**
7.	Blood Urea(mg/dl)	33.04 ± 1.08	33.56 ± 1.75 NS	35.31±1.37 NS
8.	Serum Creatinine (mg/dl)	0.94 ± 2.07	0.95 ± 3.60 NS	0.93±2.07 NS
9.	Plasma Glucose (mg/dl)	90.06 ± 1.08	174.9±13.06**	216.5±11.07**
10.	U. Albumin (mg/l)	13.86 ± 0.65	19.63 ± 1.03**	18.60 ± 0.98** ns
11.	U. Creatinine (mg/kg/day)	13.77 ± 0.67	14.58 ± 0.67 NS	14.23 ± 0.63 NS ns
12.	Cr. Clearance (ml/min)	113.93±4.52	113.94±4.51 NS	104.59± 3.47 NS ns

Values are expressed as mean ± s.e.m..

Comparison vs control: [NS = non significant ($p>0.05$), * = significant ($p<0.05$), ** = highly significant ($p<0.01$)]

Comparison vs type 1 DM: [ns = non significant ($p>0.05$), Δ = significant ($p<0.05$), Δ Δ = highly significant ($p<0.01$)]

Urinary Albumin was analyzed by using a reversed-phase C5 column with ultraviolet detection. HPLC separation of the sample solution was performed on a discovery bio wide pore C5 column and detected by ultraviolet absorbance at 280 nm.

Procedure

Aliquots of urine samples were stored at –20°C until testing. Before analyzing for albumin by HPLC, the samples were thawed and gently mixed by vortexing. The sample solution then injected into the HPLC system and albumin elutes as a sharp peak at approximately 2.8 min with an overall runtime of 8 min.

The concentrations of albumin in urine were determined by comparing the peak areas of albumin in test (unknown concentration) with peak areas of albumin in known concentration standard solutions with the following formula:

RESULTS

Patients with type I diabetes were younger ($p<0.05$) and had increased duration of diabetes ($p<0.05$) while patients with type 2 diabetes were obese ($p>0.01$). Significant difference in mean duration of diabetes were found between both type I and type II diabetes ($p<0.05$). Patients with diabetes (type I and II) had significantly increased mean fasting plasma glucose and significantly lower serum albumin level ($p<0.01$). A non-significant difference was observed in blood urea, serum creatinine, urinary creatinine and in creatinine clearance ($p>0.05$) while excretion of mean urinary albumin was significantly increased in diabetic patients (type I & II) compared to control subjects (Table 1).

DISCUSSIONS:

Albumin in urine can be estimated by various methods[14, 15] . Traditionally, the dipstick test was used to detect protein in the urine. However, testing albuminuria by dipstick is semiquantitative, and insensitive. It can not detect albuminuria reliably in the range of <300 mg/d. Thus, dipstick test could only estimate albumin in urine in the concentration of 300 mg/24 hours and above [16] . The first analytical test that could measure lower albumin concentration was a radioimmunoassay using iodine labeled albumin which is based on immune reaction[17] . However, this method is time consuming, tedious and too expensive for routine laboratory measurement [18] . Therefore other immnuno based immunonephelometry and immunoturbidimetry automatic assays have been developed where the albumin containing sample (urine or serum) is mixed with albumin antibody, resulting in small aggregates. These aggregates will scatter light and the amount of scatter is measured [19]. Though these methods are also based on recognition of albumin by antibodies, are relatively cheap and have the ability to detect small amount of albumin in the range of microalbuminuria these immunoreactive methods however estimate only complete albumin molecules[20]. After discovery of HPLC it is found that immnuno based methods has limitations that it can not detect all albumin present in the urine. That portion of albumin which can not be measured by immnuno based methods is known as immunounreactive albumin[21]. Measurement of urinary albumin by HPLC offers advantages over other methods that it detects that part of albumin also that can not be detected by other methods[22]. Thus HPLC can detect immunounreactive protein. In the present study significantly increased mean value of urinary albumin in both type 1 and type 2 diabetic patients compared to control indicated that despite excretion of albumin well with in the range of normoalbuminuria, significantly higher albumin was excreted in both types of diabetic patients than normal control subjects. Thus, suggested that it may be the immunounreactive albumin whose excretion is increased at least in the initial stages of renal insult in diabetic patients. Significantly increased albumin excretion in diabetic patients despite non-significant difference in blood urea, serum creatinine and creatinine clearance rate could be explained on the basis of natural history of chronic renal disease where development of overt nephropathy passes through five different stages[23]. Where stage I is the stage of renal hyper perfusion with preserved GFR (GFR >90 ml/min) that declined to 60-89 ml/min in stage II. Patients in CKD of stage I and stage II are generally asymptomatic and GFR estimation alone does not clinch the diagnosis. Clinical manifestation typically appears in stage IV and V. In the initial stages of CKD other evidences of kidney diseases are often required for example presence of proteinuria and heamaturia. Thus accurate measurement of protein in urine is the only way to identify those prone to develop CKD in future.

Study highlights the need for adopting the HPLC as a method for early detection of impending renal disease in diabetic patients on one hand and simultaneously it also dictate the need for defining the method specific criteria for defining the albuminuria in diabetic patients.

CONCLUSIONS:

Study found significantly raised albumin in urine of diabetic patients. Thus detection of albuminuria by HPLC may aid identification of impending renal failure in patients with diabetes even before condition was diagnosed by conventional methods.

Limitations:

Though collection of 24 hours of urinary sample and estimation of albumin by HPLC were the strengths of our study, study has certain limitations. We did not estimate urinary albumin by radioimmuno assay techniques thus direct comparisons between two techniques were not made. That demands estimation of urinary albumin by both techniques (HPLC vs radioimmunoassays) to be able to determine fraction of unreactive albumin excretion over different range of albumin excretion.

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