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PROGNOSTIC SIGNIFICANCE OF FLUORESCAMINE LABELED SERUM ALBUMIN AND GLOBULIN VALUES IN PATIENTS WITH DIABETES MELLITUS RESULTS IN END STAGE RENAL DISEASES

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ABSTRACT

Proteinurea is considered to be major prognostic factor in patients with certain chronic diseases, such as cancer and ESRD. High serum albumin, gamma globulin, beta-2 & alpha-1 microglobulin is common in patients with diabetes mellitus; nevertheless, the relationship between proteinuria and diabetes mellitus prognosis has not been verified. The mean arterial pressure was 30±9%. The mean serum albumin and globulin was 186.9±0.5mg/day. Patients with and without high urinary albumin levels were similar in age, because of diabetes mellitus. The survival rate of patients with proteinuria was 50.41% and 80.62% in those without proteinuria($P<0.001$). The calibration curve for four specific proteins responsible for ESRD in diabetes was found linear in the range of 10^{-9} to 10^{3}nmolL^{-1}, correlation coefficient(r^2) was found to be 0.9926. The LOD and LOQ were observed as 3.28nmolL^{-1} to 23.80nmolL^{-1}. To elucidate the effect of labeled serum albumin and globulin level on prognosis of patients with diabetes mellitus results in ESRD.

KEYWORDS: albumin, globulin, diabetes mellitus, Fluorescamine, prognosis, capillary electrophoresis, end stage renal disease (ESRD).

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INTRODUCTION

Proteinurea is a common laboratory finding in patients with diabetes mellitus, occurring in approximately two third of patients. The possible causes of hypoalbuminemia in patients with diabetes mellitus are chronic inflammation, infection, malnutrition, hemodilution, proteinuria, systolic heart failure and other mechanisms [1]. By reducing colloid blood pressure, hypoalbuminemia, pulmonary congestion and heart failure can affect the diabetes mellitus. In disease states, such as end-stage chronic renal failure, cancer and infection, and in the elderly, proteinuria is associated with poor outcomes [2-4]. The importance of serum albumin, globulin levels as a prognostic factor in diabetes mellitus has not been well characterized. The primary aim of this study was to examine whether urinary globulin and albumin is an independent predictor of survival in patients with diabetes mellitus in end stage renal disease, as well as the prediction of survival of patients[5-7]. To date various techniques have been applied for protein separation and detection like HPLC-UV [8-10] GC- MS [11], CE-MS [12-16] etc. HPLC has an advantage of purifying proteins with multi-step separations, but the limitation is that it usually needs a larger sample in comparison to CE. Variation in concentration of human serum albumin, β_2, α1 microglobulin and γ globulin provides valuable information about the metabolic diseases and various diagnostic applications [17-19]. Proteinurea is defined as persistent urinary albumin excretion above 300 mg/day (20 to 200 µg/min). Human serum albumin has been reported as an indicator in renal disease such as uremia, nephritic diseases and microalbuminuria, high risk of cardiovascular diseases and diabetes [20-26]. The presence of β_2, α1 microglobulin and γ globulin concentration in urine is higher than in serum [27-30]. In diseased state of diabetes mellitus, multiple myeloma and macroglobulinemia specific serum proteins are released and identified by protein urine level. However, the magnitude of urinary protein excretion is associated with a linear increase in the risk of diabetes mellitus leads to progression of end-stage renal disease (ESRD) [31-32].

2. MATERIALS AND METHODS

2.1 Chemicals

Human serum albumin, gamma, α_1 globulin and β_2 microglobulin was purchased from Sigma-Aldrich, Steinheim, Germany (internal standard ISTD). Fluorescamine {4-phenylspiro [furan-(3H), 1-phthala] 3, 3'Dione} was obtained from Fluka, Buchs, Switzerland. NaOH and Boric Acid was from CDH India. HPLC grade methanol, and analytical grade hydrochloric acid and acetone were obtained from Ranbaxy, Ropar, India. MilliQ Water purification unit was purchased from Millipore (Bangalore, India) was used in the preparation of buffer and sample solution.

2.2 Standard Solutions

Standard stock solutions of proteins were prepared in the concentration of 100-1000 µg/ml in water. Fluorescamine in the concentration of 10 mM was prepared daily. For calibration the standard solution of four biomarkers were prepared as a mixture solution. The internal standard was prepared at a concentration of 100-60 µg/ml. Testing Serum albumin globulin levels were analyzed in patients using standard bromocresol purple dye-binding method. Proteinuria and microalbumenria were defined as the lowest quartile of albumin and globulin was < 50g/dl. Urine samples were collected from patients before meal in the morning and after 2 hours of taking the meal. Additional laboratory testing of proteins released in urine was done separately using labeling of proteins with Fluorescamine in capillary electrophoresis.

2.3 Capillary electrophoresis analysis

Analysis was performed on Prince-C 255 capillary electrophoresis instrument with programmable injector and high voltage source (Prince Technologies, The Netherlands). Separations were carried out at 25 kV applied voltage. Fused silica capillary with internal diameter of 75 µm, 55 cm total length and 30 cm effective length, was purchased from Composite Metal Services Ltd. (Worcestershire, UK). Samples were introduced hydro dynamically by applying 40

mill bar pressure for 12 sec. For fluorescence detection, an ARGOS 250B instrument (Flux Instruments, Switzerland) was used where the excitation light was filtered through a Schott glass UG-11 filter and a 495 nm cut off filter was applied for the limited light. The voltage used for photomultiplier tube (PMT) was 800 Volts. Fresh capillary was charged by rinsing with 0.1 M sodium hydroxide for about 40 min followed by rinsing with water for 15 min followed by reconditioning with buffer.

RESULTS AND DISCUSSION

ANALYTICAL & CLINICAL METHOD

In the present work, direct detection proteins was done through labeling of biomarkers i.e., human serum albumin and β_2, α_1 microglobulin, γ globulin are reacted with varying amounts of fluorescamine. It was found that 15mM of fluorescamine concentration was sufficient to achieve the optimum peak area. At higher reagent concentration, the peak area decreased due to fluorescence quenching by one of the hydrolysis products of fluorescamine. The reaction mixture became turbid when higher concentration of reagent was added to the reaction mixture and attempts solubilize the reagent by addition of acetone also decreased the peak area. Under optimized condition in the present work, the reaction is completed within 15 min. Effect of fluorescamine concentration on human serum albumin, β_2,α_1 microglobulin and γ globulin are shown in Fig1.

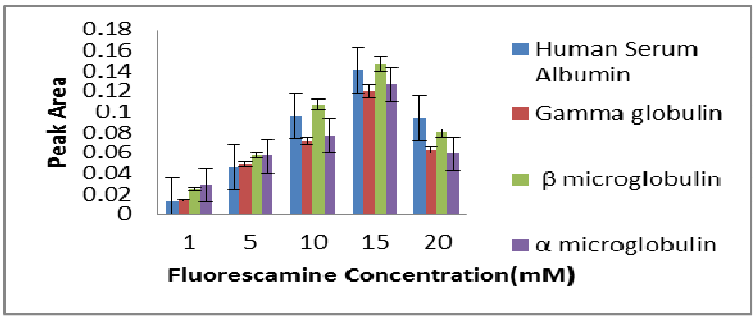

Figure 1

Effect of fluorescamine concentration on derivatives of proteins Operating conditions and peaks identification as a = human serum albumin; b = gamma globulin; c =β_2 microglobulin; d = α_1 globulin

3.1 Optimization of the derivatization conditions

Peak area values of human serum albumin and gamma globulin suggested that the maximum yield is achieved only when they are reacted with a buffer of pH 9.0. Buffers of pH range 8.0-9.5 are tried but the borate buffer of 30 mM ionic concentration gives the best results and it was further used for subsequent studies. Reaction pH study is shown in Fig 2.

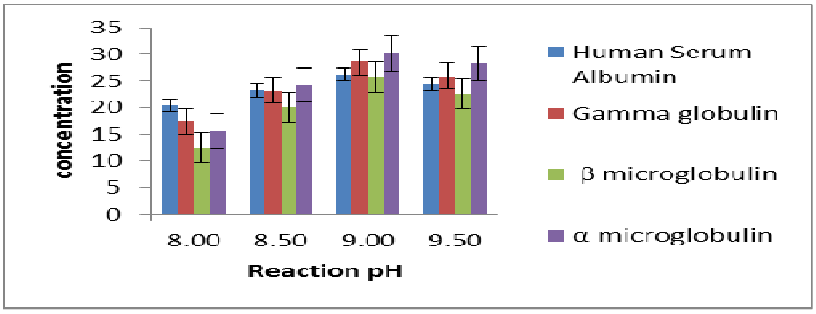

Figure 2

Effect of pH on fluorescence yield. Capillary, 55 cm (30.0 cm effective length)× 75 μm I.D. BGE, borate buffer of pH 9.0 containing 20% MeOH Applied voltage was 20 kV. Peaks, * = electro-osmotic flow; a = human serum albumin; b = gamma globulin; c =β2 microglobulin; d = α1 globulin

3.2 Optimization of separation conditions

It was clear from previous studies that protein is highly absorbed in silica capillary separated with borate buffer of 9.0 pH and was used in the analysis of human serum albumin , β_2 microglubulin, α_1 and γ globulin in addition to it 1 M NaOH was flushed in capillary between the two runs. Both of the proteins migrate at same migration time so an organic modifier ACN was used first to resolve the human serum albumin, gamma, alpha globulin later methanol was tried, which gave better results. For baseline resolution a 20% MeOH was selected.

3.3 Method validation

3.3.1 Calibration curve, linearity and detection limit

Calibration curve were plotted between the concentration $1x10^{-9}$ to $1x10^{3}$ M of human serum albumin and β_2, α_1 microglobuline, γ globulin and found to be rectilinear over the range. The correlation coefficient (r^2), limit of detection (LOD) and limits of quantification (LOQ) are shown in Fig 3 and table 1.

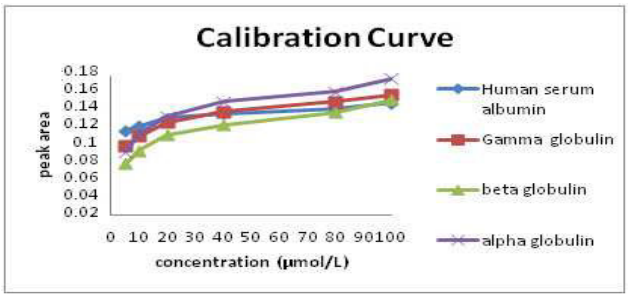

Figure 3
Calibration curve for quantification and linearity of four biomarkers

Table 1
Regression data for all the analytes.

Protein	Intercept (c^b)	Slope(m^a)	Corrélation coefficient (r^2)	LOD[c](nmol L^{-1})	LOQ[d] (nmolL^{-1})
HSA	0.0159	12885	0.9926	4.18	13.89
Λ	0.02808	31362	0.9980	7.27	23.80
β-2	0.0173	21675	0.9975	6.32	20.32
α-1	0.0165	11744	0.8979	3.98	16.54

($y = m^a x + c^b$) x = concentration, mol L^{-1}; y = peak area; c^b = intercept; m^a = slope; calibration graph constructed over ten concentration levels; results are the averages of three replicate analyses.
[c] LOD = limit of detection (S/N = 3), [d]LOQ = limit of detection (S/N = 10).

3.3.2 Precision

The precision of analytical method was determined by comparing the retention time of protein standard. Table 2 represents interday and intraday repeatability in terms of percentage RSD in retention time and peak area. Inter day repeatability was measured within 30 days in Table 2.

Table 2
Interday and intraday Precision of biomarkers by proposed analytical method

Protein	Intraday[a] [RSD[b] (%)	Interday[a] [RSD[b] (%)]
HAS	0.72	6.28
Λ	0.42	5.30
β-2	0.53	4.60
α-1	0.67	5.89

[a]All results are the averages of six replicate analysis, [b]R.S.D= Relative Standard Deviation

3.3.3 Recovery

The extraction recoveries were determined by comparing the corrected peak areas of proteins extracted from spiked urine samples with that of un-extracted standard containing the same amount of proteins. For the determination of proteins in urine three replicate analyses of samples spiked at the concentration of 25 and 50 µmol/L were carried out. The same procedure for the sample preparation and derivatization as described in the previous section. Table 3 represents the average recovery ranged 90.9-110.4%.

Table 3

Evaluation of accuracy and precision of the proposed method in biological matrix

Proteins Concentration	**25 µmol L^{-1}**		**50 µmol L^{-1}**	
Proteins	**Found (µmolL^{-1}) ± SD[1]**	**Recovery ±RSD[2] (%)**	**Found (µmolL^{-1})±SD[1]**	**Recovery ± RSD[2] (%)**
HAS	23.4 ± 0.0011	93.6 ± 3.82	45.2 ± 0.0015	90.4 ± 4.85
Γ	24.8 ±0.003	99.2 ± 4.57	51.1±0.0037	102.2 ± 5.20
β2	22.3±0.005	89.2±2.98	48.6±0.0026	96.0±3.26
α1	25.6±0.008	100.2±1.67	39.4±0.0046	78.8±2.99

[1] ***Averages of six replicate and their 1. SD (Standard Deviation) 2. (Relative Standard Deviation)***

3.4 Clinical Utility and analysis of Biomarkers in patients

Albumin, globulin and patient characteristics

The clinical utility of the biomarkers need to be determined preferably in real samples and patient's conditions. Early detection of biomarkers must detect disease before it is clinically apparent. This usually requires measuring the biomarkers in repository samples from a retrospective longitudinal faction of apparently healthy subjects who were monitored for the development of the disease. By comparing data from patients who developed the disease with age-matched control subjects, a screen-positive rule is established and then used to determine whether the biomarker detects early disease before it is clinically obvious. Patients were in the age group of 27-60 years, and average period of suffering from Diabetes is 6±2 years. And Proteinurea was more common in women and those with diabetes mellitus. High urinary albumin was also associated with higher levels of C-reactive protein, blood urea, and creatinine. Patients with high urinary albumin were likely to be more prone to ESRD rather patients without ESRD as shown in (Table 4).

Table 4

Baseline characteristics of the group based on presence or absence of proteinuria (globulin, albumin ≤300 mg/day)

	Total no of patients (N = 350)	**Albumin < 300 mg/day (without ESRD) (n = 70)**	**Albumin >300mg/day (with ESRD) (n = 280)**	**P value**
Age (years)	±50	52 ± 10	57	0.60
Body weight (kg)	±27	≥27	≤27	0.036
Diabetes (%)	78	57	69	0.02
Hypertension (%)	45	42.64	41.75	0.93
Systolic pressure (BP)mmHg	±148	≥148	<148	0.31
Diastolic pressure(BP)mmHg	± 90	47 ± 87	36± 87	0.3 4
Laboratory				
Albumin (mg/day)	186.9 ± 0.5	143.0 ± 0.7	127.2 ± 0.1	0.05
Serum sodium (mmol/L)	139 ± 4	135 ± 5	136± 3	0.07
Blood urea (mg/dL)	78 ± 31	65 ± 21	59 ± 20	0.003
Creatinine (mg/dL)	2.5 ± 2.0	2.5 ± 1.3	1.2 ± 2.0	0.021
Hemoglobin (g/dL)	14.6 ± 1.0	13.2 ± 1.2	18 ± 1.0	0.02
Total cholesterol (mg/dL)	145 ± 50	142 ± 30	170 ± 41	0.003
C-reactive protein (mg/L)	4.1 ±3.2	10.5±1.4	5.6± 7	0.002
Hemodynamics Mean blood pressure (mm Hg)	72 ± 10	71 ± 10	72 ± 10	0.11

Diabetes % of average patients: Effected by the release of proteins(albumin and globulin) in different no of patients having with or without end stage renal disease.

Albumin and motility

High urinary albumin and globulin was an independent predictor of all-cause mortality in diabetes mellitus associated with ESRD, multivariable Cox regression analysis, shows that body weight has no interaction with serum albumin as shown in Table 5. After stratification of the population into subgroups based on body weight, systolic and diastolic blood pressure etc., Proteinurea remained significantly associated with increased mortality of patients suffering from diabetes mellitus with end stage renal disease. However, this study presents facts that changes in levels of proteinuria are prognostic of ESRD development and decline in GFR in people with Diabetes mellitus. Detection of these proteins as a biomarker by fluorescamine shows the validation of method in patient's urine as an early diagnostic technique.

Table 5

All-cause mortality in patients with Diabetes and ESRD having high albumin, globulin compared to those without high albumin, globulin

	Serum albumin level (g/dL)		P value
	Proteinuria (≤300)	No proteinuria (>300)	
Number (total=350)	70 (72.2 %)	280 (27.8 %)	
1 y Mortality (total = 50)	24 (35.29 %)	26 (14.28 %)	<0.002
Multivariate* HR (95% CI)	2.8 (1.4-3.7)	1.5 (reference)	<0.002
Age- and sex-adjusted HR (95% CI)	4.6 (1.0-3.1)	1.0 (reference)	<0.003

HR, hazard ratio; CI, confidence interval.

****Multivariate adjusted for BMI category, demographics, diabetes, serum sodium, hemoglobin.***

CONCLUSION

Patients with diabetes mellitus with proteinurea have a greater risk of mortality compared to those without proteinurea, even after having multiple detection techniques for prognostic factors. This concludes that serum albumin and globulin level as a simple biomarker that could be considered as a predictor of increased mortality in patients with diabetes mellitus ends in end stage renal disease. A prospective screening study was performed on normal individuals and on those who screened positive. The aim of this test is to establish whether the disease is detected early enough to make a clinical difference.

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