

# A sensitive immunoblotting method for screening of microalbuminuria in diabetic patients' urine

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## ABSTRACT

**Objective:** Urinary albumin excretion is a useful marker in the prognosis of diabetic nephropathy and microvascular diseases. Methods such as enzyme linked immunosorbent assay (ELISA), radio immunoassay (RIA), radial immunodiffusion, albu screen, micro bumin and micral test are usually used for detection and screening of microalbuminuria in these patients. With consideration to the cost of an assay, methods such as ELISA and RIA are not suitable methods for screening purpose. Therefore, the aim of this work is to set a dot immunoblotting method for the measurement and screening of microalbumin in urine samples.

**Methods:** The study was conducted during the period August 2001 to June 2003 at the National Research Center for Genetic Engineering and Biotechnology (NRCGEB) and Pars Hospital Laboratory of Tehran, Iran on 96 diabetic patients' urine samples. First, anti human albumin antibodies (Abs) were produced in rabbit and immunoglobulin G (IgG) fraction was purified by protein-A affinity chromatography. Titer of Abs and optimum incubation conditions were tested by direct ELISA. Then different concentration of human albumin (0-300 mg/l) was loaded to nitrocellulose membranes and

was assayed by dot immunoblotting method. The specificity and cross reactivity of Abs was tested by SDS-PAGE electrophoresis and western immunoblotting. The sensitivity of the method was calculated from human albumin calibration curve and compared with commercial immunoturbidimetric assays.

**Results:** Our results indicates that in using IgG with the concentrations 0.5-1 µg/ml ( $2 \times 10^{-5}$  to  $10^{-4}$  dilutions) the intensity of color directly increased with the increase of human albumin standards in blots. Western immunoblotting of urine samples did not show any cross reactivity with other urine proteins. Comparison of results of this method by commercial immunoturbidimetric methods indicates the correlation regression of approximately 0.979. The sensitivity of the method was approximately 5mg/L of human albumin.

**Conclusion:** This simple immunoblotting method could measure microalbumin in urine. This method is more suitable for screening of microalbumin in diabetic patients' urine with a lower test cost.

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Microalbuminuria is defined as persistent elevation of albumin in urine of 30-300 mg/day or 20-200 µg/min. Microalbuminuria continues to receive attention in patient with non-insulin dependent diabetes mellitus (NIDDM) and hypertension.<sup>1</sup> It deserves attention as microalbuminuria has emerged as an important risk factor for left ventricular hypertrophy, myocardial infarction, stroke, peripheral vascular diseases and

retinopathy independent of blood pressure.<sup>2-4</sup> Microalbuminuria may be a useful measurement during pregnancy and appears particularly in women who develop pre-eclampsia during pregnancy.<sup>5,6</sup> In all of the above cases, microalbuminuria is a useful marker of generalized endothelial cells dysfunction and mortality in NIDDM patients related to its renal and cardiovascular sequelae.<sup>7,8</sup>

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The albumin values (20-300 mg/day) are less than the values detected by routine urine dipstick testing, which does not become positive until protein excretion exceeds 300-500 mg/day, therefore, finding a suitable laboratory method with high specificity and sensitivity and low economic cost for screening of microalbuminuria is important in clinical laboratories.<sup>9</sup>

Radio-immunoassay is still the most common method in measuring microalbumin, but many different methods such as enzyme linked immunosorbent assay (ELISA), immunoturbidimetric, latex immunoassay, liposomal immunoassay and micral test have been described.<sup>10-14</sup> However, some problems must be considered when using these procedures that have acceptable sensitivity, such problems are as follows, variation of urine's pH and unknown chemicals in urine, which affect in these aqueous manners. These problems has influence on laboratory results and requests and following up of microalbumin in insulin dependent diabetes mellitus (IDDM) patients.<sup>15</sup>

With increasing diabetic patients worldwide, in under developed societies it is not possible to screen microalbuminuria by ELISA and RIA methods. Therefore, we have developed a simple dot immunoblotting method for measuring and screening of microalbuminuria in Iranian diabetic urine samples. The use of new and non-commercial anti human albumin antibodies, the optimum conditions of the assay such as time and temperature of incubation and concentration of antibodies and cross reactivity were also studied.

**Methods.** The study was conducted during the period August 2001 to June 2003 at National Research Center for Genetic Engineering and Biotechnology (NRCGEB) and Pars Hospital Laboratory of Tehran, Iran on 96 diabetic patient's urine samples. Immunization, production and purification of antibodies (Abs) was carried out as the method described by Gurvey.<sup>16</sup> Polyclonal anti human albumin Abs was produced in rabbit by injection of human serum albumin (Iranian Blood Transfusion Product) in a hyper immunization program. The  $\gamma$ -globulin fractions of rabbit serum were precipitated by 33% saturated ammonium sulfate for 3 times at 4°C. Pellet was dissolved in phosphate buffer saline (PBS, 0.1 M pH=7.4) and dialyzed for 2 days. After that, immunoglobulin G (IgG) fractions was purified by protein-A sepharose (Calbiochem) immunoaffinity chromatography.

The titer of Abs was estimated as the method, which was defined by Mohamed et al.<sup>10</sup> Briefly, 96 micro well (Nunc Inc.) were coated by 200 mg/L of human albumin, in carbonate buffer (0.05 M, pH=9.6) at 4°C for 16-24 hrs. Then micro wells

were blocked by 5% skim milk (Biotech) in Tris-HCl buffer (50 mM, pH=7.4) for 30 minutes. After washing for 3 times by Tris buffer, anti human albumin (1st Abs) was added in different dilutions (up to  $3 \times 10^{-8}$ ) and incubated for 2 hrs at 22-25°C. After washing 3 times, hoarse radish peroxidase conjugated goat anti-rabbit IgG (2nd Abs.) (Calbiochem) was pipetted and incubated for 60 minutes at 22-25°C again. Finally, after washing 3 times, o-phenylenediamine (OPD) substrate (Calbiochem) was added for 30 minutes and reaction was stopped by sulfuric acid (1M). The absorbance was measured at 450 nm with ELISA reader (Multi Scan).

Dot immunoblotting was carried out as the method described by Gordon and Billing.<sup>17</sup> Briefly, 5  $\mu$ L of different concentration (0 up to 300 mg/L) of human albumin standards directly were loaded on 0.45  $\mu$ m nitrocellulose membranes (Schleicher and Schuell). After air drying, the membranes were blocked by skim milk (5%) for 30 minutes. Then 1st Abs was added in different concentrations (1 ng/ml up to 100  $\mu$ g/ml). Then membrane was incubated for 60 minutes at room temperature. Then the membrane was washed 3 times by washing buffer. The 2nd Abs was added for 60 minutes. Finally, after washing 3 times, the membrane was developed by 4-chloro-naphtol substrate (Bio-Rad). Reaction was stopped by removing the substrate and washing the membrane.

To assay the urines of diabetic patients, 5  $\mu$ L of 24 hours or random urines was loaded on the membranes directly and immunoblotting was carried out as the method, which was described for albumin standards.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblotting of urine proteins, total protein of urines was precipitated by 12% tri-chloro acetic acid (TCA) for 30 minutes at 4°C. After centrifugation, the pellets were dissolved in 100  $\mu$ L of sample solvent. The SDS-PAGE was carried out on 13% resolving gel based on the buffers used by Laemmli.<sup>18</sup> Each sample was loaded to 3 separate gels and electrophoresis was carried out by LKB-pharmacia electrophoresis system. After running, one of the gels was stained by comasi blue and the 2nd one was stained by silver staining as the method described by Dunn.<sup>19</sup> The 3rd one was transferred to nitrocellulose membrane with semi dry transfer system (LKB pharmacia) and immunoblotting was carried out by the method which was described by Elkon.<sup>20</sup>

Immunoturbidimetry was carried out with the procedure manual of randox microalbumin detection kit (Randox). Briefly, 100  $\mu$ L of urines was added to the assay buffer and incubated for 5 minutes at 37°C. Absorbance ( $A_1$ ) was measured at 340 nm. Then 100  $\mu$ L of antibody solution was added and

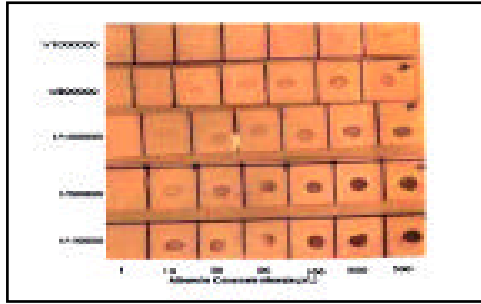


Figure 1 - Human serum albumin (0-300 mg/L) was tested with different dilutions of rabbit anti-human albumin antibodies ( $10^{-6}$  to  $10^{-4}$ ) by dot immunoblotting method.

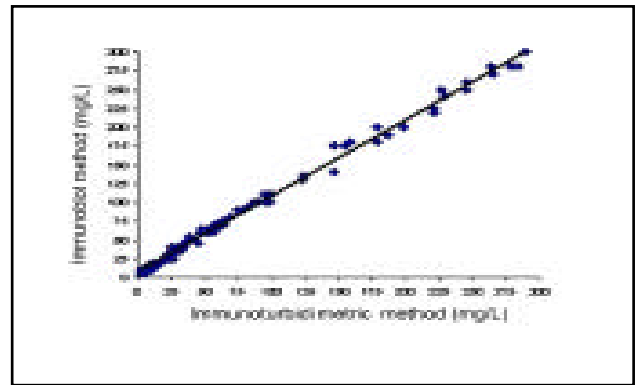


Figure 4 - A comparison of the dot immunoblotting and immunoturbidimetric methods in measuring microalbuminuria. (N=96,  $r=0.979$ , slope=1.0139, Y-intercept=6.7 mg/L).

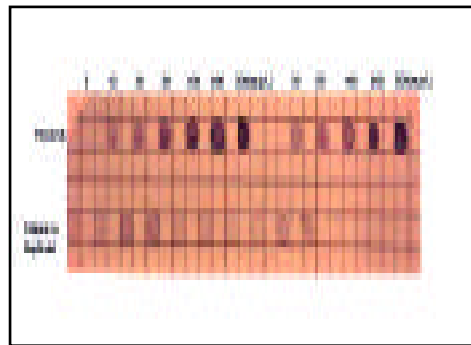


Figure 2 - Dot immunoblotting of patient's urine samples. Urine samples of insulin dependent diabetes mellitus patients were tested by dot immunoblotting method as the albumin standards.

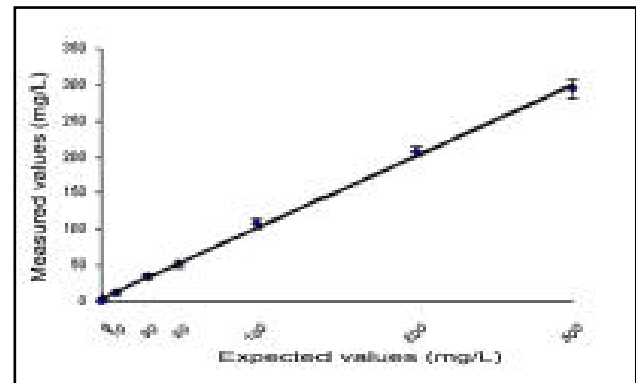


Figure 5 - Standard calibration curve of human albumin standard by dot immunoblotting method. (Y-intercept=3.5 mg/L and zero standard values  $\pm 1$  SD=0.8 mg/L, slope=1.008, n=4).

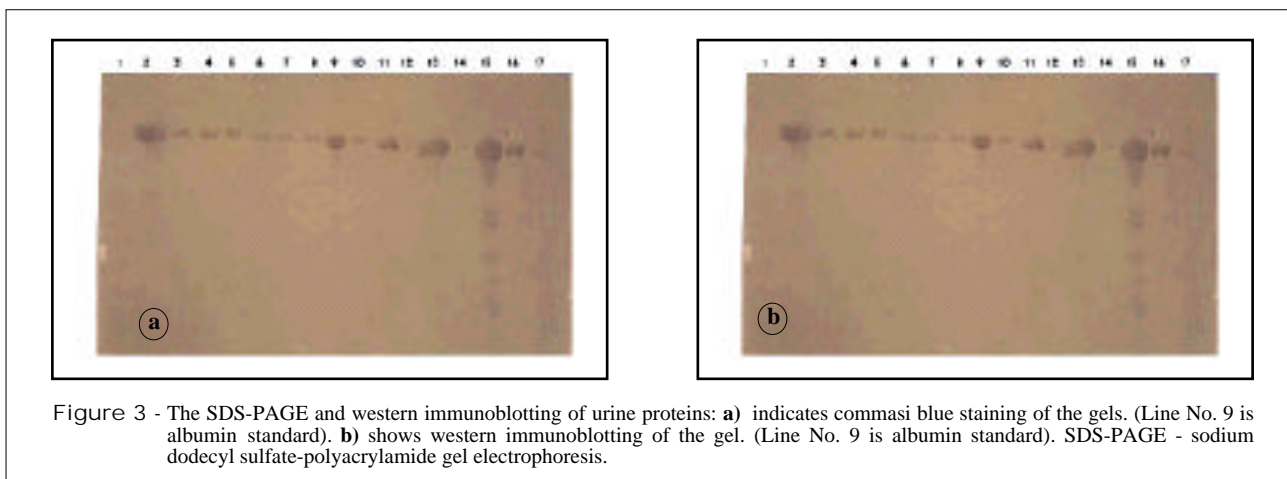


Figure 3 - The SDS-PAGE and western immunoblotting of urine proteins: **a)** indicates commasi blue staining of the gels. (Line No. 9 is albumin standard). **b)** shows western immunoblotting of the gel. (Line No. 9 is albumin standard). SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

incubated for 15 minutes. Final absorbance ( $A_2$ ) was measured and the change in absorbance ( $A_2-A_1$ ) calculated. Microalbumin concentration was calculated from the change of absorbance by using albumin standard curve.

Statistical analysis using excel program was used to analyze the correlation regression, Y-intercept and slope of the calibration curve. The sensitivity was estimated by the zero standard value plus 2.5 standard deviation of zero standard value in the calibration curve.

**Results.** Rabbit anti human albumin was used for detection of human albumin in direct ELISA manner. The results of albumin standard curve showed a linear pattern up to 250 mg/L of albumin while the titer value of Abs was approximately  $10^{-7}$  dilution. The same incubation condition in ELISA was tested to set dot immunoblotting. Different concentration of human albumin (0-300 mg/L) was loaded to nitrocellulose membrane. Then different dilutions of 1st Abs. ( $10^{-4}$  -  $3 \times 10^{-7}$ ) was used for optimum color intensity. In  $10^{-4}$  and  $2 \times 10^{-5}$  dilutions, the intensity of blot color was increased by increasing the albumin concentration in a linear manner **Figure 1**. The urines and standards were loaded on nitrocellulose membrane in duplicate and dot immunoblotting was carried out using  $10^{-4}$  dilution of anti human albumin Abs (**Figure 2**). Results show that microalbumin in urines was detectable in lower albumin concentration ( $<10$  mg/L).

The SDS-PAGE and western immunoblotting of urine proteins were carried out to evaluate the specificity and cross reactivity of Abs, which was used in dot immunoblotting. Urines from 15 patients were selected for this purpose. After cold TCA precipitation of urine total proteins, the equal volume of each urine sample was loaded in 3 separate gels as described above. **Figure 3** shows the comassi blue staining and western immunoblotting of these samples. Silver staining shows urine that contains many protein bands but in western immunoblotting only the band of albumin was interacted with Abs (**Figure 3b**). This indicate the specific reaction and any cross reactivity of these Abs, which was used in dot immunoblotting.

To validate the accuracy of this method, 90 diabetic patient's urine samples were tested with immunoturbidimetric and dot immunoblotting manner. As **Figure 4** shows, correlation regression of the 2 methods was approximately 0.979 ( $r = 0.979$ ) and Y-intercept of the assay was approximately 6.7 mg/L.

Sensitivity of the method was determined using albumin standard curve. For this purpose, different concentration of human albumin standard (0-300 mg/L) was tested by immunoblotting method. **Figure 5** indicates the standard calibration curve by

dot immunoblotting. The Y-intercept is approximately 3.7 mg/L (estimated zero standard value) with 0.8 mg/L standard deviation. Estimated sensitivity of this method was approximately 6 mg/L of human albumin in urine samples.

**Discussion.** The validity and performance of any quantitative assay can be evaluated using 3 basic approaches.<sup>21</sup> In the first case, the reproducibility of the calibration data or standard curve can be assessed by measuring parameters that describe the curve. A second approach is to employ a set of test samples containing various levels of the analyte to determine the reproducibility of the concentrations. Finally, both the estimates of test samples concentrations and the reproducibility of the calibration can be used to measure assay performance.

In this work, we opted to use the simplest approach using calibration data and urine samples reproducibility as a measure of assay optimality. The rationale was that, once the conditions necessary for an optimal condition of human albumin standard and urine samples are determined.

According to the definition of titer, the dilution was approximately  $10^{-7}$  gave an absorbance over 0.25 once daily and this indicates efficient immunization and high affinity of these antibodies.

In dot immunoblotting method, urine microalbumin is detectable with an acceptable sensitivity and specificity in a semi-quantitative manner.

The number of patients with significant chronic failure is rising in developed countries and diabetic hypertensive patients should be carefully monitored for the development of renal insufficiency using screening tools.<sup>22</sup> Therefore, due to the increasing prevalence of diabetes and hypertension in our society, monitoring of nephropathy in these patients is necessary. Due to high cost of ELISA and RIA microalbumin kits, these methods are not suitable for screening purposes in underdeveloped countries. The results and experiences of this work indicate that dot immunoblotting method is a useful tool for screening purposes. The minimum requirement in the use of expensive facilities such as ELISA reader or -counter or turbidimeter proves to be the advantage of using this method in screening of microalbuminuria. Finally, this immunoblotting method is a suitable laboratory procedure for screening of microalbuminuria in diabetic patients.

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