

## Ultrasensitive time-resolved immunofluorometry of human albumin in urine using monoclonal antibodies—a new assay for microalbuminuria

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**SUMMARY.** We describe a two-site, sandwich methodology for human albumin in urine. In the assay, albumin binds to a solid-phase monoclonal antibody and to another monoclonal that is biotinylated. The immunocomplex is then quantified by adding streptavidin which is labelled with an europium chelator, using time-resolved fluorometry. The assay is extremely sensitive ( $< 1 \mu\text{g/L}$ ) and specific. A sample predilution of 251-fold or more is needed before analysis. The analytical parameters studied (precision, recovery, linearity, comparisons) were found to be satisfactory. The assay is simple to perform and is proposed as a non-isotopic alternative to radioimmunoassay for the quantification of small amounts of albumin in urine for the purpose of assessing microalbuminuria.

*Additional key phrases: europium chelates; biotin-streptavidin*

A high percentage of insulin-dependent diabetics excrete abnormal amounts of albumin in urine<sup>1,2</sup> and this has been found to be a predictor of a highly increased risk of renal failure and early mortality.<sup>3-8</sup> The proteinuria of diabetics can decrease in response to several therapeutic modalities and the early detection of microalbuminuria may lead to prevention or at least delay of diabetic nephropathy.<sup>9,10</sup>

Dipstick methods for microalbuminuria lack clinical sensitivity and specificity.<sup>11</sup> The most suitable procedures for assessing microalbuminuria are immunological.<sup>12-14</sup> In this report, we describe a new highly sensitive and specific two-site non-competitive method for quantification of albumin in urine which is based on a time-resolved fluorescence system that has been recently described.<sup>15-17</sup>

### MATERIALS AND METHODS

#### Instrumentation

For solid-phase time-resolved fluorometric measurements, we used the Model 615<sup>®</sup> Immunoanalyzer (CyberFluor Inc., Toronto, Canada) which also performs data reduction.

#### Materials

The europium chelator 4,7-bis (chlorosulphophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA) was synthesised as described elsewhere.<sup>18</sup> Streptavidin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St Louis, USA.  $\text{EuCl}_3$  hexahydrate was obtained from Aldrich Chemical Co., Milwaukee, USA and sulphosuccinimidyl-6-(biotinamido)-hexanoate (NHS-LC-biotin) from Pierce Chemical Co., Rockford, USA. All other chemicals were purchased from Sigma. White, opaque 12-well microtitre strips (Immulon II) were products of Dynatech Labs, Alexandria, USA.

The coating buffer was 50 mmol/L Tris solution, pH 7.80. The blocking buffer was 50 mmol/L Tris solution, pH 7.80, containing 9 g of NaCl, 10 g of BSA and 0.5 g of sodium azide per litre. The assay buffer and the sample diluent were the same as the blocking buffer. The streptavidin-europium dilution buffer was a 50 mmol/L Tris solution, pH 7.80, containing 9 g of NaCl, 40 g of BSA, 0.5 g of sodium azide, and 40  $\mu\text{mol}$  of  $\text{Eu}^{3+}$  per litre. The wash solution was 9 g/L NaCl solution containing 0.5 mL of Tween 20 per litre.

#### Human albumin standards

A stock solution of human albumin, 80 g/L, was

purchased from American Dade, Aguada, Puerto Rico 00602. The certified concentration of albumin was verified with a biuret total protein method. This solution was serially diluted in the assay buffer to obtain a series of standard solutions of the desired concentration. Typically, we used standard concentrations of 0, 5, 20, 50, 100 and 200  $\mu\text{g/L}$  to construct calibration curves.

#### *Monoclonal antibodies*

We purchased, from Medix Biochemica AB, Kauniainen, Finland, two newly developed monoclonal antibodies for human albumin, purified by protein A affinity chromatography. One antibody (Code 6501) was used for coating, the other (Code 6503) was used for detection.

#### *Specimens*

We obtained 24-h urine collections from individuals who were being investigated for various renal abnormalities. The specimens were stored at 4 °C for no more than 4 weeks until assay. Just before assay, the urines were well mixed and then centrifuged. An aliquot of urine was then diluted as described below and used for the assay. Thirty-one samples were obtained from a mixed population of apparently healthy subjects for estimation of a reference range.

## PROCEDURES

#### *Biotinylation of the detection antibody (Code 6503)*

The antibody solution (1 mL, containing 1 mg of antibody) was dialysed twice against 5 L of isotonic saline (9 g/L NaCl solution) at 4 °C. We then diluted the dialysand with an equal volume of 0.5 mol/L carbonate buffer, pH 9.1. To this solution we added 2 mg of NHS-LC-biotin dissolved in 50  $\mu\text{L}$  of dimethyl sulphoxide and incubated the mixture for 2 h at room temperature. The solution was then dialysed twice against 5 L of 9 g/L NaCl solution at 4 °C for 24 h. For the assay, we diluted the stock antibody solution 200-fold in the assay buffer (working antibody solution). The stock antibody solution is stable for at least 6 months and the working antibody solution for at least 2 weeks at 4 °C.

#### *Preparation of the streptavidin-thyroglobulin-BCPDA-Eu<sup>3+</sup> tracer*

The preparation of BCPDA-labelled streptavidin-protein conjugates is detailed elsewhere.<sup>17,18</sup> The concentration of streptavidin in the working solution of BCPDA-labelled

streptavidin-thyroglobulin conjugate was 0.30 mg/L; that of EuCl<sub>3</sub> was 40  $\mu\text{mol/L}$ .

#### *Coating microtitre wells*

The coating antibody solution was prepared by diluting the monoclonal human albumin antibody (Code 6501) in the coating buffer to a concentration of 5 mg/L. We then added 100  $\mu\text{L}$  of the antibody solution into wells of microtitre strips and allowed the antibody to adsorb at room temperature overnight. The wells were then washed twice with the wash solution prior to adding 200  $\mu\text{L}$  of the blocking buffer per well and incubating for 1 h at room temperature. Before use, the wells were washed twice with the wash solution.

## COMPARISON METHODS

We used two methods for urinary albumin determination: (a) the albumin double antibody kit available from Diagnostic Products Corporation (DPC), Los Angeles, USA; and (b) the albumin RIA kit available from Pharmacia, Uppsala, Sweden. Both kits were used as recommended by the manufacturers.

#### *Assay procedure*

All urine samples were diluted with the assay buffer, 251-fold, to obtain solution A. A convenient dilution is 20  $\mu\text{L}$  of urine plus 5 ml of diluent. Urines containing greater than 50 mg/L of albumin were further diluted 51-fold. A convenient dilution is 0.1 mL of solution A plus 5 mL of diluent. Before the assay, the strips were washed twice with the wash solution. One hundred  $\mu\text{L}$  of standards and diluted urine samples (in duplicate) were pipetted into each well. The strips were then shaken in an automatic shaking device for 120 min and washed six times with the wash solution. One hundred  $\mu\text{L}$  of the working biotinylated antibody solution was then added to each well ( $\sim 250$  ng antibody/well), the wells were then incubated for 30 min with shaking at room temperature and washed six times as above. One hundred  $\mu\text{L}$  of the working tracer reagent (streptavidin-BCPDA-Eu<sup>3+</sup>) was then added. After incubation at room temperature for 30 min with shaking, the strips were washed six times with the wash solution and dried with a stream of air. Surface fluorescence is measured using a CyberFluor 615 Immunoanalyzer which has an automatic data reduction capability. The results along with the calibration curve are printed automatically as

soon as the readings are complete (about 5 min for a 96-well plate).

## RESULTS

### Assay optimisation

We have used both available antibodies for either coating the solid-phase or for detection and we found no significant difference in the results. We have also tested a number of different proteins or macromolecules in the composition of the assay buffer. These included bovine serum albumin (BSA) ovalbumin, bovine thyroglobulin, horse serum, polyvinylpyrrolidone, and gelatin. We found no detectable cross-reactivity of BSA with the antibodies used and selected to use it as a convenient carrier protein in the assay buffer. The time courses of the three incubation steps were studied. The first incubation step was selected to be 2 h at room temperature with shaking. Increasing the incubation further resulted in less than 5% increase in signal per hour. The second and third incubation steps reached equilibrium values in 30 min at room temperature with shaking.

When using two monoclonal antibodies in two-site sandwich-type immunoassays, it is possible to combine the antigen binding step and the detection antibody binding step. This can be done by simultaneously pipetting the sample and the biotinylated antibody in the antibody coated microtitration wells. In the proposed assay configuration we have selected to incubate the antigen first, then add the biotinylated antibody after washing. This was done because we wanted to avoid the high-dose hook effect which is present at high antigen concentrations.<sup>19</sup> Calibration curves were constructed by a protocol that involves sequential or simultaneous antigen and detection antibody solution addition and are shown in Fig. 1. It can be seen that in the sequential addition protocol, the fluorescence increased gradually as the dose increased from 0 to 500  $\mu\text{g/L}$  and then reached a plateau which was maintained even at doses up to  $10^6 \mu\text{g/L}$ . When a simultaneous addition protocol was used, fluorescence peaked at a dose of 500–1000  $\mu\text{g/L}$  but then started to decline gradually reaching background levels at a dose of  $10^6 \mu\text{g/L}$ . The high-dose hook effect is due to the fact that at

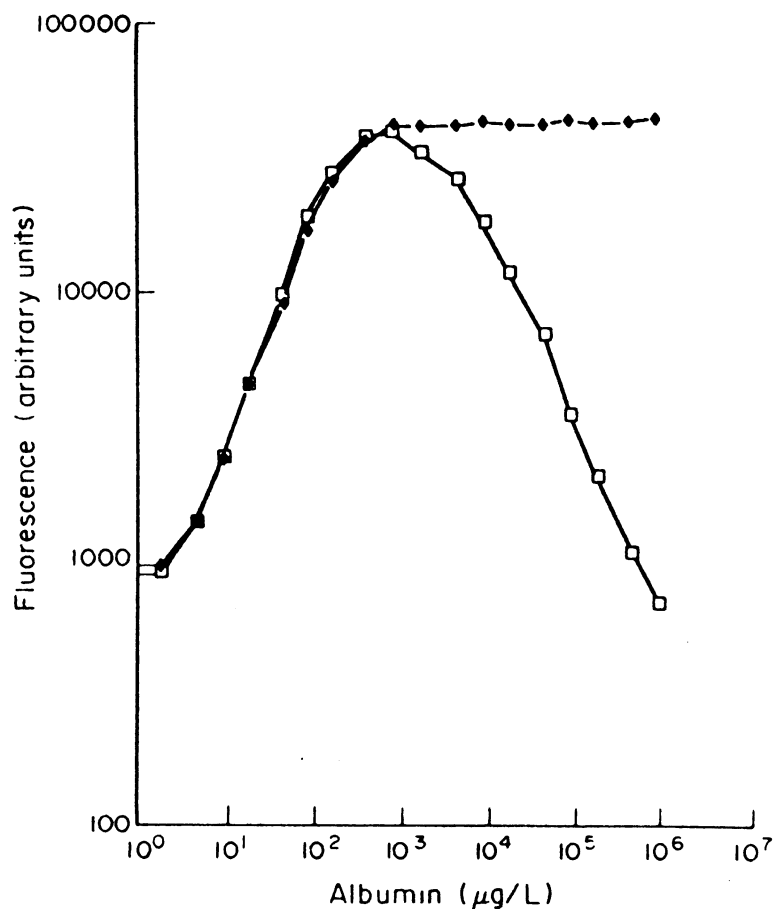


FIGURE 1. Dose-response curves for the proposed assay. (□) The sample and biotinylated antibody were pipetted simultaneously into the antibody coated wells to demonstrate the high-dose hook effect. (◆) The protocol described in the text was followed; the biotinylated antibody was added after sample incubation and washing.

very high antigen concentrations, there is not enough detection antibody to form a sandwich of the type: solid-phase antibody-antigen-detection antibody; the antigen saturates the solid-phase but most of the detection antibody is exhausted and washed out after binding to the excess antigen present. As can be clearly seen from Fig. 1, the high-dose hook effect was completely eliminated by using a sequential incubation protocol with washing before the biotinylated antibody addition.

## ASSAY EVALUATION

### *Detection limit and precision*

The detection limit of the proposed assay defined as the albumin concentration corresponding to the mean plus three standard deviations of the zero standard signal for 24 replicates was found to be  $\leq 1 \mu\text{g/L}$ . The within-run precision was studied by analysing control solutions at three concentrations (10.8, 41.8 and 158.7  $\mu\text{g/L}$ ) 24 times each. The coefficients of variation (CVs) observed were 4.6, 4.1 and 9.1 %, respectively. The between-run precision was studied by analysing control solutions at three levels (14.3, 29.6 and 77.3  $\mu\text{g/L}$ ), 18 times each, over a period of 2 weeks. The CVs observed were 9.8, 7.4 and 8.7 %, respectively.

### *Dilution test*

We evaluated the dilution linearity of the procedure by assaying samples serially diluted with the assay buffer. The dilution ranged from 25-fold to 3200-fold. Linear dilution curves were obtained in all cases. The proposed general dilution in this assay is 251-fold or more depending on the concentration of albumin in urine.

### *Analytical recovery*

This experiment was performed by the addition of known concentrations of exogenous albumin to aliquots of prediluted urine that was assayed before the additions to calculate the initially

present concentration. Six urines were used with two additions to each (50 or 95  $\mu\text{g/L}$ ). Recovery ranged from 92 to 106 % with an average of 99.3 %.

### *Specificity*

We have examined the specificity of the antibodies used in this study for a number of proteins from other animal sources. A bovine serum albumin solution 100 g/L, an ovalbumin solution, 100 g/L, undiluted horse serum (total protein 61 g/L) and undiluted rabbit serum (total protein 59 g/L) gave no detectable signal in the proposed assay. Bovine  $\gamma$ -globulin and mouse IgG gave apparent cross-reactivities of 0.00025 % and 0.006 %, respectively. From these data, we conclude that the antibodies used are very specific for human albumin. We did not study cross-reactivities with other human proteins because, due to the high-sensitivity of our assay, the results would be invalidated by even ultratrace amounts of albumin which is invariably present in reagent-grade protein preparations. The comparison data with two established techniques (see below) suggest that our assay is specifically quantifying albumin in urine.

### *Reference range*

We have analysed, by the proposed procedure, 24 h urine samples obtained from a mixed population of 31 apparently healthy individuals. Some statistical parameters for the proposed procedure and the two widely used radioimmunoassays and the suggested reference range are shown in Table 1. These data are in broad agreement with reference ranges suggested by other investigators.<sup>8,20-22</sup>

### *Correlation with other methods*

We compared results obtained with the present method and two established radioimmunoassay procedures. The results are summarised in Table 2. There is good agreement between the proposed and the comparison methods.

TABLE 1. Statistical data for 31 urine specimens from normal individuals

	Albumin (mg/24 h)		
	Present method	DPC, RIA	Pharmacia, RIA
Mean	4.9	4.9	5.5
Median	4.5	4.5	5.4
Minimum	0.3	0.6	0.5
Maximum	12.8	14.9	14.9
Proposed reference range	< 13	-	-

TABLE 2. Linear least squares regression correlation of microalbumin results obtained with the proposed assay and other methods

	Albumin range (mg/24 h)		
	0-15	0-200	0-8000
IFMA (y) vs DPC(x):			
Slope	0.88	1.01	0.93
Intercept	0.76	0.91	43
r	0.92	0.98	0.94
N	31	51	71
IFMA (y) vs Pharmacia (x):			
Slope	0.85	1.01	0.91
Intercept	0.18	-1.3	32
r	0.97	0.98	0.92
N	31	51	71

IFMA, immunofluorometric assay.

## DISCUSSION

For the assessment of microalbuminuria qualitative and semiquantitative methods are not acceptable because of the poor sensitivity and the subjectivity of the results obtained. Thus, many false positives and false negatives may be observed even with newer methods that claim improved analytical sensitivity.<sup>11</sup> Immunological approaches for quantifying small amounts of albumin in urine are preferred because they offer good sensitivity, precision, and ease of performance.<sup>12-14</sup> The widely used methods are based on polyclonal antibodies, competitive principles, and radioactive labels. Recently, monoclonal antibodies that react with different epitopes on the albumin molecule became available and they offer a means of devising non-competitive, two-site assays which have a number of advantages over competitive techniques.<sup>23</sup> On the other hand, non-radioactive labels like enzymes, chemiluminescent, or fluorescent probes are now starting to replace radionuclides because of the well known disadvantages of radioactivity. In this report, we describe a method that is simple to perform and combines the advantages of using monoclonal antibodies in a non-competitive assay design. The detection system used is based on labeled streptavidin and has been thoroughly described.<sup>15-17</sup> We propose that this assay has good analytical characteristics and is suitable for the assessment of microalbuminuria in clinical laboratories.

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