Enzyme Immunoassay for Urinary Albumin

B. A. Fielding, D. A. Price, and C. A. Houlton

We describe an enzyme-linked immunosorbent assay for urinary albumin, performed on microtiter plates with use of commercially available antisera and peroxidase conjugate. The assay range is 3–1000 µg/L, the sensitivity 625 pg. The method is suitable for measurement of albumin excretion in either normal or pathological urine. For 20 normal children, the range of urinary albumin excretion was 1.7–22.9 mg/24 h.

Additional Keyphrases: proteins · pediatric chemistry · enzyme-linked immunosorbent assay

Following the advent of more sensitive techniques for the assay of urinary albumin, there has been increased clinical interest in differentiating subtle abnormalities in urinary albumin excretion. For example, exercise-induced albuminuria has been measured to define early glomerular changes in diabetic subjects (1, 2), and patterns of albumin and low-molecular-mass protein clearance after renal transplantation have been studied (3, 4).

Most current methods for sensitive albumin measurement are radioimmunoassay (5–7) involving 125I-labeled albumin. Disadvantages of using this reagent include its short shelf life, its associated health hazards, and the expense of equipment used to measure gamma-emitting isotopes. Radial immunodiffusion (8) and electrophoresis (9) have been used but are rather cumbersome and slow. An immunoturbidimetric assay has recently been reported (10), but this would not be sufficiently sensitive to measure the lowest concentrations of urinary albumin in healthy children as reported in this paper.

In response to the need for a simple assay sensitive enough to distinguish normal concentrations of urinary albumin from slightly increased concentrations, in a range sufficiently large to accommodate excretion rates in pathological states, we have developed an enzyme-linked immunoassay (ELISA) for the measurement of urinary albumin. In common with other ELISA techniques (11, 12), this procedure overcomes many of the disadvantages of radioimmunoassay. It is sufficiently sensitive to measure accurately albumin at low concentrations, is easily set up in a laboratory, and is very simple to carry out.

Materials and Methods

Reagents. Human transferrin and immunoglobulin G (IgG), o-phenylenediamine, Tween 20, and bovine serum albumin were obtained from Sigma Chemical Co., London, U.K. The following antisera and peroxidase conjugate were obtained from Miles Laboratories Ltd., Slough, U.K.: rabbit anti-human albumin (lyophilized), cat. no. 65-051; goat anti-human albumin (liquid), cat. no. 61-015; and horseradish peroxidase (EC 1.11.1.7) conjugated to anti-goat IgG (heavy + light chain), cat. no. 61-201. Inactivated rabbit serum was obtained from the Wellcome Foundation Ltd., London, U.K.

Buffers. A solution of phosphate-buffered saline and Tween 20 (PBS-Tween) was prepared by dissolving 7.88 g of Na2HPO4·2H2O, 2.04 g of NaH2PO4·2H2O, 9.00 g of NaCl, and 0.5 mL of Tween 20 in 1 L of distilled water.

Diluent was prepared by dissolving 2.5 mg of bovine serum albumin per liter of PBS-Tween. At this concentration, the bovine serum albumin did not cross react with the antiserum in the assay system but was sufficient to inhibit nonspecific binding.

The coating buffer (0.5 mol/L carbonate/bicarbonate buffer, pH 9.6) was made by dissolving 1.59 g of Na2CO3, 2.93 g of NaHCO3, and 0.20 g of NaN3 in 1 L of distilled water.

The substrate solution was prepared just before use: to 25 mg of o-phenylenediamine in 100 mL of 0.2 mol/L phosphate solution, pH 6.0 (28.6 g of Na2HPO4 in 1 L of distilled water), 160 µL of 300 g/L hydrogen peroxide solution was added. The substrate solution was prepared in and dispensed from a brown bottle.

Standards. Human albumin, dissolved in the diluent solution to give a stock solution of 200 mg/L, was kept at −20 °C. From this, standards were made up in diluent at concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200, and


CLIN. CHEM. 29/2, 355–357 (1983)
1000 μg/L. Fresh standards were prepared every three days.

Antisera. The "first antibody" (Figure 1) was diluted 10,000-fold in coating buffer. The "second antibody" and the peroxidase conjugate were diluted 10,000-fold in PBS-Tween. These dilutions could be kept at 4°C for as long as two weeks.

Equipment. The microtiter plate reader was the Microelisa® minireader (Dynatech Laboratories Ltd., Billingshurst, West Sussex, U.K.). The microtiter plates were Nunc-Immunoplates from Gibco Europe Ltd., Middlesex, U.K.

Samples. We obtained 24-h urine collections from healthy school children under normal conditions of life. Adequacy of collection was confirmed by comparison of estimated creatinine excretion with measured creatinine excretion (13). Inadequate collections were not included in the study. Aliquots (2 mL) of urine were stored at −20 °C with 20 μL of inactivated rabbit serum added to help prevent adsorption of albumin to the storage tubes. For the assay, samples were diluted 250-fold with the diluent solution.

Assay procedure. The assay is basically a modified double-antibody sandwich ELISA as described by Voller et al. (14). The antibody-antigen complex on which the assay is based is given in Figure 1. All incubations were carried out at room temperature. We coated the wells of the microtiter plate by adding 200 μL of the first antiserum solution to each well and incubating for 1.5 h. The outside wells were not used, to avoid possible "edge effects." After emptying the wells and washing them three times with PBS-Tween, we added 200 μL of standard or diluted sample in duplicate to the coated wells and incubated for 1 h. We repeated the washing procedure, then added 200 μL of the second antiserum solution, which contained 1 mL of inactivated rabbit serum per 100 mL to inhibit any nonspecific binding due to cross reactivity with components of the first antiserum. After a 1-h incubation and another washing, we added 200 μL of the peroxidase conjugate solution, and let this incubate for 1 h. After washing again, we added 200 μL of substrate solution to each well at timed intervals. We incubated the plate in the dark for 30 min, then added 50 μL of 1 mol/L hydrochloric acid at timed intervals. The absorbance of the contents of the wells was read at 490 nm with the microtiter plate reader.

Results

Standard curve. The standard curve is shown in Figure 2.

![Diagram of antibody-antigen complex](Image)

**Fig. 1. Diagram of antibody-antigen complex**

![Absorbance curve](Image)

**Fig. 2. Standard curve and results for serial dilution of normal urine**

The assay sensitivity, defined as the least amount of albumin that is significantly different from zero at the 95% confidence limit, is consistently 3.125 μg/L, the concentration of the lowest standard. This is equal to 625 pg of albumin per well and equivalent to 0.781 mg of albumin per liter in a urine sample that has been diluted 250-fold.

Precision. "Within-run" precision (Table 1) was estimated by assaying two urine samples 30 times in one assay. The "between-run" precision was estimated by assaying them in 12 consecutive assays.

Specificity and accuracy. We checked the specificity of the assay system with human IgG and transferrin, 1 mg/L and detected no cross reactivity. The analytical recovery of albumin from a diluted urine sample was between 95% and 104% (Table 2).

Samples. The mean albumin excretion in 20 normal children, ages six to 18 years (mean 9.5 years), was 7.6 mg/24 h, with a range of 1.7–22.9 mg/24 h.

Discussion

The range for albumin accurately detected by this ELISA, 3–1000 μg/L, is sufficiently broad to measure accurately.

**Table 1. Precision of the Method**

<table>
<thead>
<tr>
<th>Albumin, μg/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Between run (n = 12 each)</td>
<td>4.42</td>
<td>0.39</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>20.75</td>
<td>1.91</td>
<td>9.2</td>
</tr>
<tr>
<td>Within run (n = 30 each)</td>
<td>4.35</td>
<td>0.17</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>21.75</td>
<td>0.87</td>
<td>4.0</td>
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**Table 2. Analytical Recovery of Albumin**

<table>
<thead>
<tr>
<th>Added Albumin, μg/L</th>
<th>Measured Albumin, μg/L</th>
<th>Recovery, %</th>
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<tbody>
<tr>
<td>25</td>
<td>25.9</td>
<td>104</td>
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<tr>
<td>50</td>
<td>47.4</td>
<td>95</td>
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<tr>
<td>100</td>
<td>98.9</td>
<td>99</td>
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normal and abnormal concentrations of urinary albumin. The sensitivity of the assay, 625 pg per microtiter well, is as good as or better than that quoted for radioimmunoassay (5–7) and superior to that of a turbidimetric assay (10). The main advantage of this method is that only commercially available reagents are used. Moreover, the reagents are inexpensive, and each antiserum is used at such a high dilution that, in theory, 100 000 single determinations could be made from 2 mL of neat antiserum. The assay could be converted for the assay of other urinary proteins with the same enzyme conjugate. Although the method is fairly lengthy (5–6 h) each step takes only a few minutes and results may be obtained within a day.

The range of albumin excretion we found in 20 normal children, 1.7–22.9 mg/24 h, compares well with previously reported values in adults of 2.2–12.6 (7), 8.6–12.6 (6), 3.6–14.1 (15), 8.9–21.9 mg/24 h (16), and 16 mg/24 h (17). Little information is available on the albumin excretion rate of children, and normal values for different ages, height, and weight require definition and will be established in our laboratory.

We thank Mrs. K. Cordwell and the Salford Department of Medical Illustration for manuscript preparation; G. M. Addison, Z. Niewola, D. B. Gordon, and J. Lane for technical advice; and the children of Higher Lane and St. Bernadette’s schools. Financial support was received from the Wellwood Kidney Foundation and The North Western Regional Health Authority.

References