Rapid, Competitive Enzymoimmunoassay for Albumin in Urine

J. Chesham, S. W. Anderton, and C. F. M. Kingdon

In this solid-phase competitive enzymoimmunoassay for albumin in human urine, antiserum to human serum albumin labeled with horseradish peroxidase (EC 1.11.1.7) is incubated with solid-phase-bound human serum albumin in the presence of sample or standard. Results obtained correlate well (r = 0.96) with those of an established fluoroimmunoassay. The present assay covers the range 0.9 to 200 mg/L and can be performed within 1 h. These characteristics, together with the simplicity of the assay protocol, make it very useful for monitoring low concentrations of albumin in urine. Detection of such minimal albuminuria allows initiation of therapy that may prevent development of clinical proteinuria and associated diabetic nephropathy.

Additional Keyphrases: albuminuria • proteinuria • diabetes mellitus • nephropathy

In diabetes mellitus, the strains imposed on the kidney by the widely fluctuating concentrations of blood glucose can, and frequently do, lead to increased urinary excretion of protein, which is symptomatic of diabetic nephropathy. The onset of this proteinuria develops gradually over a period of years, and excretion rates for albumin slowly increase from normal, 2-20 mg/24 h (1), to manyfold this rate. This increase in urinary albumin has been linked to higher mortality rates (2); however, strict diabetic control in the early stages of proteinuria can reduce or remove the symptoms and arrest the development of nephropathy (2).

Various methods have been described for quantifying low concentrations of albumin in urine, including radial immunodiffusion (3) and "rocket" immunoelectrophoresis (4), two slow and inaccurate techniques. More recently, highly sensitive radioimmunoassays (RIA) have been described for quantifying albumin in urine (5, 6). In 1983, Fielding et al. (7) documented a further advance: they developed a "sandwich"-type enzyme-linked immunosorbent assay for albumin, overcoming the disadvantages of short shelf-life, health and safety hazards, and equipment expense associated with RIA. However, their assay has several disadvantages. It takes 6 h to perform and involves four incubation steps. In addition, urine samples must be diluted before being applied to the assay plate. In contrast, we describe here a competitive enzymoimmunoassay (cEIA) that yields accurate and reproducible results within 1 h, involves only three incubations, and does not require sample predilution. In addition, the assay procedure is considerably simpler than that used in the "sandwich" type of assay (7, 8).

Materials and Methods

Apparatus

We used Nunc® certificate grade 96-well microtiter plates, from Inter-med, Kastmup, Denmark. For colorimetric analysis we used a Tittertek MCC/340 multispectrophotometer (Flow Laboratories, Ayrshire, Scotland), linked to an Apple Ille data-processing system. Fluorescence was monitored in a Model 204-A fluorescence spectrophotometer (Perkin-Elmer, Beaconsfield, U.K.).

Reagents

Human serum albumin (Cohn Fraction V), horseradish peroxidase (EC 1.11.1.7), o-phenylenediamine, 1-fluoro-2,4-dinitrobenzene, sodium hydrogen carbonate, sodium periodate, ethylene glycol, and sodium borohydride were supplied by the Sigma Chemical Co., Poole, U.K. "Sephacryl" S-300, a high-resolution gel chromatography matrix, was purchased from Pharmacia Ltd., Milton Keynes, U.K. Polyclonal sheep antiserum specific for human serum albumin was from International Laboratory Services, London, U.K. All other reagents were of "Analar" grade, from BDH Chemicals Ltd., Dagenham, U.K.

Buffers and Standards

Coating solution, pH 9.6: Dissolve 8.5 g of NaCl, 1.0 g of human serum albumin, and 0.2 g of Na3PO4 in 1 L of distilled water. Adjust the pH to 9.6 with 5 mol/L NaOH solution. This reagent is stable for at least three months, when stored at 4 °C.

Assay diluent, pH 7.5: Dissolve 8 g of NaCl, 0.2 g of KH2PO4, and 2.8 g of Na2HPO4 · 12 H2O in 1 L of distilled water, and add 500 µL of Tween 20 (polyoxyethylene sorbitan monolaureate) dispersing agent.

Substrate solution: To 75 mL of substrate buffer pH 5 (5 g of citric acid and 17 g of Na2HPO4 · 12 H2O in 1 L of distilled water) add 40 mg of o-phenylenediamine and 25 µL of H2O2 (300 mL/L solution).

Standards: Prepare a 1 mg/mL standard by dissolving 0.1 g of human serum albumin and 0.85 g of NaCl in 100 mL of distilled water. Check the albumin content by measuring the absorbance at 280 nm, which should read 0.54 (±0.1) A. Dilute this to prepare 1, 2, 10, 50, 100, and 200 mg/L standards.

Assay Protocol

Apply coating solution to all the wells of the microtiter plate, 150 µL per well. Cover the plate and leave it at room temperature for 15 min, then thoroughly wash the plate in assay diluent. Add sample or standards to the wells (15 µL

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per well). Then add anti-human albumin antiserum—peroxidase conjugate, prepared according to the method of Nakane and Kawaoi (9) except that we recommend purifying the conjugate on Sephacryl S-300. Gently tap the plate to ensure mixing of each well's contents. After incubation for 30 min at room temperature, again thoroughly wash the plate in assay diluent. Apply freshly prepared substrate solution to all the wells, 150 μL per well, and allow substrate oxidation to proceed for 10 min in the dark at room temperature. Terminate the reaction by adding 150 μL of 1 mol/L H2SO4 solution per well, and measure the absorbance of the wells' contents at 492 nm.

The fluoroimmunoassay used in the correlation study involves incubating standard or sample with anti-human serum albumin in the presence of fluorescein isothiocyanate labeled albumin. After 2 h at room temperature a precipitating antibody is added and incubated for a further 1 h. The incubation mixture is centrifuged and the washed pellet monitored for fluorescence (E690).

Results

Assay characteristics. The standard curve in Figure 1 was generated by using the cEIA system described. The mean coefficient of variance (CV) for quadruplicates of these standards was 3.0% (range 0.7–4.4%). The sensitivity, as measured by the 99% confidence limit of the zero standard (18 replicates), was <0.9 mg/L, the working range of the standard curve being 0.9–200 mg/L. Intra-assay measurement of albumin was precise within this concentration range, the mean CVs for both intra- (2.9%) and interassay (7.6%) analysis being acceptable (Table 1). Analytical recovery of human albumin added at 10, 20, 50, 100, and 200 mg/L averaged 100.38% (range 96.8–104.4) (Table 2).

Correlation of cEIA with fluoroimmunoassay. We examined 36 presumably normal urine samples by the cEIA and a competitive fluoroimmunoassay that incorporated a fluorescein isothiocyanate-labeled albumin conjugate. Correlation between the two assays was good (r = 0.96) over a range of albumin concentrations tested (Figure 2).

Discussion

The importance of monitoring low concentrations of albumin in urine as a predictor of proteinuria development is becoming more and more apparent (1, 2, 10). Therefore, a simple quantitative method for monitoring the concentrations of human albumin excreted would be of great value. Although enzyme immunoassays have been previously described for measuring albumin in urine (7, 8), the methodologies involved restrict their usefulness. In contrast we have developed a competitive enzyme immunoassay, based on an antibody to human serum albumin conjugated to peroxidase. The assay is extremely simple to perform and yields accurate, reproducible results within 1 h. The results obtained upon screening 36 presumably normal individuals demonstrate the assay's usefulness for detecting abnormal concentrations of albumin in urine. A single urine specimen was found on examination by cEIA to be outside the normal range (2–20 mg/L); this result was confirmed by the fluoroimmunoassay.

By using precoated plates, total assay time can be reduced. Further, by using an unconventionally high concentration of coating, the requirement for blocking the poly styrene is removed. The system described does not suffer from nonspecific binding problems that usually affect sensitive enzyme immunoassays. By obviating predilution of the urine samples, imprecision through errors in pipetting is reduced. Because the urine is diluted during the course of the assay, however, specimen quality and concentration do not appear to affect assay precision.

<table>
<thead>
<tr>
<th>HSA, mg/L</th>
<th>Added</th>
<th>Recovered</th>
<th>Recovery, %</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>9.85</td>
<td>99.0</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>20.30</td>
<td>102.0</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>48.45</td>
<td>98.8</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>104.30</td>
<td>104.0</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>199.40</td>
<td>99.7</td>
<td></td>
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</tbody>
</table>

* Different volumes of 1 g/L HSA solution in physiological saline was added to fresh urine to yield the added concentrations listed.

Fig. 1. Standard curve for human albumin (HSA) in urine

![Graph](image)

Fig. 1. Standard curve for human albumin (HSA) in urine

![Graph](image)

Fig. 2. Correlation in the cEIA results with those by fluoroimmunoassay (FIA) in 36 supposedly normal patients

Table 1. Imprecision Data

<table>
<thead>
<tr>
<th>Sample</th>
<th>HSA, mg/L</th>
<th>CV, %</th>
<th>HSA, mg/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.0 ± 0.04</td>
<td>2.7</td>
<td>1.45 ± 0.13</td>
<td>8.97</td>
</tr>
<tr>
<td>2</td>
<td>2.7 ± 0.02</td>
<td>2.3</td>
<td>2.85 ± 0.24</td>
<td>8.42</td>
</tr>
<tr>
<td>3</td>
<td>9.1 ± 0.02</td>
<td>3.7</td>
<td>9.2 ± 0.62</td>
<td>6.7</td>
</tr>
<tr>
<td>4</td>
<td>16.5 ± 0.01</td>
<td>2.6</td>
<td>16.8 ± 1.10</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Results are mean ± SD.
Distribution of Paraoxon Hydrolytic Activity in the Serum of Patients after Myocardial Infarction

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The activity of paraoxonase in serum was found to be bimodally distributed, both in a control group and in a group of patients who had suffered myocardial infarction. Activity in the myocardial infarct group was significantly lower than in the control group. Low paraoxonase activity in serum may provide an indication of susceptibility to the development of coronary heart disease.

Aldridge 1 classified esterases into two groups according to their interaction with organophosphate anticholinesterases. "A"-esterases hydrolyze such compounds; "B"-esterases are inhibited by them. The A-esterase enzyme(s) capable of hydrolyzing the organophosphate paraoxon (O, O-diethyl-O-p-nitrophenyl phosphate) is called paraoxonase (EC 3.1.1.2; aryloesterase).

The distribution of values for paraoxonase activity in human serum has been studied by workers in several countries. In studies conducted on more than 90 individuals, independent surveys in Britain (2), Germany (3), Denmark (4), and North America (5–7) have all produced evidence for a bimodal distribution of this activity. In one study of 799 individuals a trimodal distribution is claimed (8), and the same conclusion is drawn in a smaller study by the same workers (9). In these two later studies, however, the workers measured residual cholinesterase activity after incubating paraoxon with serum, a method that does not take into account any components of serum other than paraoxonase that can hydrolyze paraoxon, e.g., albumin (10). Genetic studies suggest that these distributions are determined by two common alleles at a single autosomal locus (2, 5), and interest has developed in the use of this enzyme as a genetic marker in human populations.

Biochemical studies of paraoxonase in the serum of humans (and sheep) have shown that much of this activity is associated with high-density lipoprotein (HDL) (11). Recent work indicates that HDL has an important role in the removal of cholesterol from arterial walls, and consequently in protection against the development of atherosclerosis (12–14). Thus, in the context of atherosclerosis, the serum paraoxonase assay may provide a useful alternative to the standard procedure for the estimation of HDL, which relies upon determination of cholesterol (15), which in turn is very much a function of dietary state.

The validity of certain conclusions drawn in earlier studies on paraoxonase in human serum is somewhat debatable. In particular, the pH used in some assays far exceeded the physiological, and will cause rapid and variable chemical hydrolysis of the substrate (16); others did not include Ca++, a cofactor for paraoxonase (17) in the assay medium. The present study has two aims: (a) to re-examine the distribution of paraoxonase in humans under assay conditions that more closely reflect physiological conditions than do those used in previous studies, and (b) to see whether serum paraoxonase and coronary artery disease are inversely related in a group of hospital patients.

Materials and Methods

Chemicals. Calcium chloride was obtained from Hopkin & Williams, Eastleigh, Hants, England. Paraoxon (O, O-diethyl-O-p-nitrophenyl phosphate) was from Sigma (London) Chemical Co., Poole, England. All other chemicals were of AR grade.

Sources of sera. Blood was collected unheparinized from

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