Microplate Measurement of Urinary Albumin and Creatinine

Ralph A. Magnotti, Jr., Gregory W. Stephens, Rick K. Rogers, and Amadeo J. Pesce

We describe microplate methods for measurement of human urinary albumin (HUA) by competitive enzyme-linked immunosorbent assay (ELISA) and creatinine with a modified commercial enzymatic kit. Incorporation of substrate mixing into the competitive ELISA changes the dynamic absorbance—concentration response, greatly simplifying calculations and improving sensitivity and accuracy. Measurement of creatinine in urine and plasma samples with a commercially available enzymatic kit modified for analysis by use of an inexpensive microplate reader produced values comparable in precision and accuracy to those obtained by an automated kinetic Jaffe method.

Additional Keyphrases: enzyme-linked immunosorbent assay · "kit" methods · diabetes · renal disease · clinical research methods

About 30–45% of Type I diabetics eventually develop kidney failure as a result of progressive nephropathy (1). Increased rates of urinary albumin excretion not detectable by routine clinical methods—so-called "microalbuminuria" or "pauci-albuminuria"—may indicate "incipient nephropathy," which can progress to overt nephropathy (2). Because intervention is usually ineffective for overt diabetic nephropathy (3, 4), early detection of nephropathy is essential.

HUA in the normal or above-normal ranges is commonly measured immunochemically, by ELISA (5) or RIA (6). The latter method suffers from short reagent shelf-life and radiation hazard. The various versions of the ELISA method exhibit disadvantages in reproducibility and simplicity of data analysis, often requiring specialized instruments. Because the competitive ELISA is faster and more convenient, we chose to optimize this technique for measurement of HUA.

Measurement of HUA concentration alone is not sufficient to determine increased rates of albumin excretion because the concentration varies with urine flow rate. Variation in urine flow rate may be corrected for by determination of urinary creatinine, expressing albumin as the ratio albumin:creatinine (6, 7). This avoids the inconvenience of obtaining timed urine collections. However, commonly used manual Jaffe methods (8) for creatinine are time consuming and require the use of hazardous picric acid, whereas the more accurate kinetic (9) variations require the use of expensive automated analyzers. In addition, the Jaffe method is subject to various interferences (10). Largely free of interferences, enzymatic assay of creatinine (11) allows both the ELISA of HUA and creatinine correction to be performed on an inexpensive microplate reader. Consequently, we chose to modify a commercial enzymatic kit for microplate measurement of urinary creatinine.

We report here the detailed application of these methods to the measurement of normal and above-normal amounts of albumin in urine samples.

Materials and Methods

Reagents. Diethyldimalonic acid, 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB), gelatin (60 bloom), urinary metabolite lyophilisate (lyophilized gel-filtered urine from normal men), and delipidated human serum albumin (HSA) of 99% purity was obtained from Sigma Chemical Co., St. Louis, MO 63178. Rabbit anti-human albumin peroxidase conjugate was from Organon Teknika–Cappel, Malvern, PA 19355, as a solution containing 11 mg of IgG and 4 mg of peroxidase per milliliter. Concentrated hydrogen peroxide solution (500 g/L, and containing 6 mg of sodium stannate per liter as stabilizer) was from Fisher Scientific, Pittsburgh, PA 15238. Urine creatinine standards were from Abbott Laboratories, South Pasadena, CA 91030. The enzymatic kit for measurement of creatinine ("Creatinine-PAP", no. 883263) and the lyophilized human serum creatinine calibrators—Precical, Precitrol-N, and Precitrol-A—were kind gifts from Boehringer Diagnostics, Indianapolis, IN 46250.

Buffer A. To 0.8 L of distilled water add 20 mmol of diethyldimalonic acid, 160 mmol of NaCl, 0.1 mmol of EDTA, and 1 mL of Tween 20 surfactant. After adjusting this solution to pH 7.4 with 1 mol/L KOH, dilute to 1 L with distilled water. Then add 5 g of gelatin, dissolving by gentle stirring at 37 °C. The clear solution may be used after storage at 25 °C for up to one week or, stored refrigerated, for no longer than one month. Clarify the slightly turbid refrigerated solution by warming it at 37 °C or leaving it at 25 °C for several hours.

HSA standard. Prepare a 1.00 g/L stock solution of HSA in 0.1 mol/L NaHCO₃ and store frozen in 0.1-mL aliquots in polypropylene tubes at −20 °C for no longer than a year. Check the concentration of the standard by using an absorbity (5) at 280 nm of 5.4 for a 10 g/L solution of HSA.

Coating solution, 0.2 mg/L HSA. Dilute the stock HSA standard 5000-fold with 0.1 mol/L NaHCO₃.

Conjugate solution, 1:20 000 rabbit anti-albumin–peroxidase conjugate. Dilute stock conjugate 100-fold with buffer A, then store in 0.1-mL aliquots at −20 °C. For assay, dilute the thawed conjugate 200-fold with buffer A.

Substrate, 0.5 mmol/L TMB. Prepare freshly each day. Dissolve 10 mg of TMB in 4 mL of distilled water and add to 56 mL of potassium acetate buffer (50 mmol/L, pH 4.5). Just before assay, add 6 μL of the hydrogen peroxide.

Instrumentation

Polystyrene Immulon microplates and the microplate mixer were obtained from Fisher Scientific. Microplates were read with a Model 2250 ELA reader (Bio-Rad Labs., Richmond, CA 94804). For spectrophotometric measure-
ments we used a Model PM II spectrophotometer (Carl Zeiss, New York, NY 10018); for the kinetic Jaffé assay for plasma and urinary creatinine, an ABA-100 dichromatic analyzer (Abbott Laboratories, South Pasadena, CA).

Statistical Analysis

The normalities of population distributions were determined by the Wilk–Shapiro (12) test. Geometric means and standard deviations (SDs) were calculated as the means and SDs respectively, of log-transformed data. In contrast to the convention of "±", the geometric SD is related to the geometric mean by a multiplier/divisor, or "×/±". For example, a geometric mean of 5.53 ×/± 1.71 SD describes a lower limit of 3.23 (5.53 − 1.71) and an upper limit of 9.46 (5.53 × 1.71). For a range of two SDs, the multiplier/divisor is multiplied by two, in this example a range of 1.62–18.9.

Samples

For determination of normal reference intervals, overnight timed urine specimens were collected in 15-mL polystyrene tubes by apparently healthy men and women, ages 23 to 48 years, and were stored refrigerated until assayed. Patients' urine specimens were untimed random collections.

Determination of Total Urinary Protein

Total protein in urine samples was measured by a manual turbidimetric method (13).

ELISA for HUA

Previously described ELISA methods for HUA (5, 14, 15) were modified.

1. Fill wells of Immulon 2 microplates (rows B–H) with 0.10 mL of coating solution (0.2 mg/L HSA). Incubate the covered plates for 3 h at 37 °C, then overnight (12 h) at 4 °C. Plates may then be stored at 4 °C for up to four weeks in sealed plastic bags, or washed three times with buffer A and stored for four weeks or longer before use.

2. Perform assays in duplicate, using samples of settled urine diluted in polypropylene tubes with buffer A either 100-fold (normal), 200-fold (diabetic), or 1000-fold (proteinuric).

3. Prepare HSA standards by diluting stock HSA 1000-fold with buffer A and further diluting with buffer A to give standards ranging in concentration from 0 to 1.00 mg/L.

4. Wash the coated plates four times with buffer A.

5. Add 50 μL of sample or standard to the wells of the coated plate.

6. Add 50 μL of conjugate (1:20 000) to all the wells, and incubate the covered microplate for 1 h at 37 °C.

7. Wash the microplate four times with buffer A, and add 0.20 mL of TMB substrate to each well. Then immediately place the microplate on a microplate mixer, incubating until the maximal absorbance reaches 1.5–2.0 (about 15 min).

8. Remove the plate from the mixer and measure its absorbance at 650 nm.

Calculations

HSA concentrations can be determined with a pocket calculator (Texas Instruments TI-55 II or equivalent), using the regression analysis of blanked absorbance values as a function of the logarithm of HSA concentration of the standards (30–300 ng/mL, r >0.995, n = 7).

Creatinine Assay

We determined creatinine with a commercial kit (11), "Creatinine-PAP." Volumes of reagent were modified proportionately to give a final assay volume of 300 μL containing 50 μL of sample.

Urine samples were diluted 100-fold with distilled water before assay. Fresh or thawed frozen citrated plasmas were used without dilution. For plasma assays, we included a serum creatinine calibrator (e.g., Precitrol).

Results

Assay for Urinary Albumin

Assay response. The assay displays an overall sigmoidal response typical of competitive ELISA (Figure 1). However, an approximately linear region is evident in the response of absorbance as a function of the log of albumin concentration, ranging from about 30 to 300 μg/L (n = 7, r >0.995 for 12 consecutive runs on separate days).

The detection limit is 10 ng of HSA per milliliter, or 0.5 ng per well. This limit is set primarily by the sensitivity of the conjugate (1 mol of peroxidase per mole of IgG), which yields an absorbance response of approximately 1.5 in 15 min at 1:20 000 dilution, or a response of 0.9 A in 60 min at 1:80 000 dilution. At a 1:20 000 dilution of conjugate, a half-maximal response was observed with a coating concentration of 10 ng of HSA per milliliter, reaching 90% of maximum at 0.1 mg/L. The sensitivity of the assay seemed to be proportional to the conjugate dilution from 1:1000 to 1:10 000, and became only slightly more sensitive by further decreasing either conjugate or coating concentrations.

Substrate mixing produced a significant improvement in the dynamic response of the assay, as shown in Figure 1. Without mixing, the standard curve poorly fitted either a log-linear or other simple function of concentration vs absorbance, whereas mixing changed the response to one highly correlated over a 10-fold range with a log-linear function.

The effect of buffer composition. The following buffers were evaluated in the assay: 10 mmol/L sodium phosphate,
and 20 mmol/L 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid ("HEPES"), 4-morpholinepropanesulfonic acid ("MOPS"), N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine ("Tricine"), or diethylmalonic acid. Phosphate caused heavy precipitation of the gelatin in the buffer and residual precipitation of TMB in the subsequent substrate incubation step. Except for diethylmalonic acid, the others produced various amounts of precipitation, decreased color response, high blank values, or inaccurate recoveries of albumin added to urine. In contrast, the blank values produced by diethylmalonic acid were typically <0.05 A, and the buffer could be stored at room temperature without preservatives for at least one week without development of turbidity or otherwise changing the assay characteristics.

**Use of TMB substrate.** Initial attempts to use TMB in place of o-phenylenediamine resulted in variable blank values and low color yield. This was found to be due to precipitation of the TMB and the blue TMB chromophore by anions such as chloride and phosphate. Some batches of 300 g/L ("30%") H₂O₂ also caused TMB precipitation, possibly by sodium stannate, which is added as a stabilizer. For this reason, "50%" hydrogen peroxide containing no more stannate than 6 ppm (0.03 mmol/L) was used.

TMB (0.5 mmol/L) in 50 mmol/L potassium acetate buffer did not precipitate or autoxidize significantly for several hours at room temperature. After addition of peroxide, the absorbance change at 650 nm of the TMB substrate was <0.03 A/h.

TMB and the oxidized TMB chromophore are extraordinarily stable under the assay conditions used. Blank absorbance values for a covered assay plate incubated at room temperature for 16 h increased by only 0.02, and no precipitation was observed in either blanks or samples. Assay absorbance values in the range of 1.0-1.6 A (n = 17) increased in 16 h by only 2.7% (SD 1.8%), and in the range of <1.0 A (n = 45) decreased by only 1.3% (SD 2.2%). Dry TMB dihydrochloride stored without desiccation at room temperature for two years showed no significant autoxidation.

**Precision.** Twelve untimed urine specimens with albumin concentrations ranging from 2.3 to 61 mg/L were assayed in duplicate eight times to establish within-run precision. For between-run precision, these same 12 samples were assayed in duplicate on 10 consecutive days. Within-run CVs ranged from 1.2% to 14% (mean 8.3%, SD 2.1%), between-run CVs from 6.0% to 22% (mean 16%, SD 4.5%).

**Accuracy.** We added 5.0 to 56 mg of albumin per liter to normal and diabetic urine and assayed. For normal urine, analytical recovery ranged from 61% to 109% (mean 88%, SD 18%). For the diabetic urine, the recovery ranged from 80% to 116% (mean 101%, SD 16%). Addition of threefold the normal concentration of urinary metabolite lyophili- sate caused no significant change in the standard curve. Addition of a 10-fold concentration caused an increase in values of the HSA standards ranging from 5% to 20% in the range of 0.030-0.300 mg of HSA per liter.

**Albumin in Timed Urines**

Table 1 gives normal reference intervals for men and women for HUA, albumin excretion rate, and albumin:creatinine ratio. All three are log-normally distributed by the Wilk–Shapiro test (95% confidence limit) and are correspondingly expressed as the geometric mean and SD. Urine specimens were also obtained from four renal patients exhibiting total protein excretion in the range of 85-719 mg/day. These urines were found to contain correspondingly high concentrations of albumin, ranging from 54 to 215 mg/L; albumin:creatinine ratios of these samples ranged from 7.0 to 17.4.

Table 2 shows the overall correlation between albumin, albumin excretion rate, and albumin:creatinine ratio. Creatinine correction appears to increase the correlation with albumin excretion rate only slightly relative to determination of albumin alone.

**Sample preparation.** There was no significant difference between HUA values obtained by sampling supernate from settled urine or vortex-mixed urine. However, settled urine was preferred because of possible interference from sediment with the precision of sampling.

Storage conditions for urine samples were evaluated. No significant difference was noted in HUA values for 12 urine samples ranging in concentration from 3.4 to 43 mg/L when assayed after storage for at least eight weeks at 4 °C. Significant losses of HSA were observed when these samples were frozen at −20 °C either without treatment, preriched with buffer A, or diluted with an equal volume of 1 g/L Triton X-100 surfactant in 0.1 mol/L NaHCO₃; mean recoveries were 81%, 87%, and 88%, respectively. When the samples were frozen after dilution with an equal volume of either 0.1 mol/L NaHCO₃ or 0.1 mol/L NaHCO₃ in 500 g/L ethylene glycol, an increase of HUA was observed relative to refrigerated samples, with mean recoveries of 117% (SD 22%) and 111% (SD 14%), respectively.

**Creatinine Measurement**

We measured creatinine in 21 overnight timed urine samples from apparently healthy donors and a separate set of 21 plasma samples obtained from venipuncture of apparently healthy blood-bank donors, using both Boehringer kit no. 853283, Creatinine-PAP, and an automated kinetic Jaffe procedure (9). In addition, control sera—Precitrol, Precical-A, and Precical-N (Boehringer Diagnostics)—and urine standards (Abbott Labs., N. Chicago, IL) were evaluated by both methods for creatinine.

**Assay linearity.** The standard curve was linear up to at

---

**Table 1. Normal Values for Urine Samples**

<table>
<thead>
<tr>
<th>Geometric mean (x̄ ± SD*), range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Men</strong> (n = 23)</td>
</tr>
<tr>
<td>HUA, mg/L</td>
</tr>
<tr>
<td>5.18 (2.06), 1.9-26.6</td>
</tr>
<tr>
<td>Albumin excretion rate, µg/min</td>
</tr>
<tr>
<td>4.27 (1.72), 1.7-15.9</td>
</tr>
<tr>
<td>Albumin:creatinine, mg/mmol</td>
</tr>
<tr>
<td>0.546 (1.70), 0.21-2.40</td>
</tr>
</tbody>
</table>

* Standard deviation is a multiplier/divisor (see Methods section).

**Table 2. Correlation of HUA, Albumin Excretion Rate, and HUA:Creatinine Ratio**

<table>
<thead>
<tr>
<th>y</th>
<th>x</th>
<th>n</th>
<th>r</th>
<th>Regression equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin excretion rate</td>
<td>HUA</td>
<td>48</td>
<td>0.858</td>
<td>y = 0.427x + 2.05</td>
</tr>
<tr>
<td>Albumin excretion rate</td>
<td>A:C</td>
<td>48</td>
<td>0.864</td>
<td>y = 6.46x + 1.36</td>
</tr>
<tr>
<td>Ratio</td>
<td>HUA</td>
<td>48</td>
<td>0.790</td>
<td>y = 0.0525x + 0.197</td>
</tr>
</tbody>
</table>

---

**CLINICAL CHEMISTRY, Vol. 35, No. 7, 1989 1373**
least 40 mmol of creatinine per liter (1.5 A). The slope of the assay absorbance response as a function of the concentration of the creatinine standards varied by only 2.9% over six runs on consecutive days, \( r > 0.998 \) for all plots of \( A \) vs concentration (\( n = 8 \)).

**Precision.** Within-run precision was evaluated by performing seven measurements on each of 24 urine samples (including three controls) and 24 plasma samples (including three controls) by the enzymatic method. Within-run CVs for the urines ranged from 1.2% to 6.7% (mean 4.1%, SD 1.4%). For the plasmas the range was 1.2% to 5.7% (mean, 2.7%, SD 1.2%).

Between-run precision was evaluated by measuring each of 24 urine samples (including controls) and 24 plasma samples (including controls) in duplicate on seven consecutive days by using the enzymatic method. The between-run CVs for the urines ranged from 3.1% to 13.2% (mean 7.1%, SD 2.4%). For the plasmas the range was 3.0% to 8.3% (mean 5.6%, SD 1.3%).

**Accuracy.** The accuracy of the enzymatic assay was determined against values obtained by the kinetic Jaffé method.

For urine samples, the relative accuracy for creatinine standards of 177 \( \mu \)mol/L, 530 \( \mu \)mol/L, and 884 \( \mu \)mol/L was 101.1%, 112.1%, and 124.1%, respectively, for the enzymatic method; for the Jaffé method for the respective values were 110.0%, 110.0%, and 108.6%

For plasma samples, the relative accuracy was determined against Precitol, Precical-N, and Precical-A creatinine standards (assayed by the supplier by both enzymatic and Jaffé methods) of 62 \( \mu \)mol/L, 146 \( \mu \)mol/L, and 267 \( \mu \)mol/L for enzymatic assay and 80 \( \mu \)mol/L, 177 \( \mu \)mol/L, and 301 \( \mu \)mol/L for the Jaffé method. The enzymatic assay gave values of 95.8%, 97.1%, and 91.1%, respectively, relative to the controls. The Jaffé assay gave corresponding values of 93.0%, 103.8%, and 98.3%, respectively.

**Sample storage.** No significant change in creatinine values was observed when urine samples were refrigerated for at least eight weeks, or for samples frozen at \(-20^\circ C\) for at least two months.

**Reagent stability.** Because each bottle in the kit can be used to perform 128 tests, it will usually be necessary to store the working solutions. Creatinine was measured in 16 urine samples (creatinine concentrations 3.0 to 33.6 mmol/L) by the enzymatic assay with fresh reagents and with reagents that had been stored at 4 °C for 19 days. The latter produced creatinine values for the 16 refrigerated urine samples that were changed by a mean of only –0.3% (SD 6.0%). The slope of the absorbance response as a function of the concentration of the creatinine standards changed by <3% even after the reagent solutions had aged 29 days at 4 °C.

Correlation of Enzymatic and Jaffé Methods for Creatinine

Creatinine in 21 plasma and 21 urine samples was measured in duplicate by both the enzymatic method and a standard automated kinetic Jaffé method. For the enzymatic method the creatinine values of the urine samples ranged from 2.9 to 28.0 mmol/L (mean 12.7, SD 6.7 mmol/L), and the plasma samples ranged from 43.4 to 87.8 \( \mu \)mol/L (mean 6.0, SD 12 \( \mu \)mol/L). For the Jaffé method, the creatinine values for the urine samples ranged from 3.3 to 26.0 mmol/L (mean 12.5, SD 6.1) and the plasma samples ranged from 60.1 to 118.5 \( \mu \)mol/L (mean 83.7, SD 13.9).

The correlation of the enzymatic (x) and Jaffé (y) methods for urine samples is described by the regression equation \( y = 0.911x + 0.9396 \) (n = 21, \( r = 0.997 \)). The correlation of both methods with plasma samples is described by the regression equation \( y = 1.12x + 16.8 \) (n = 21, \( r = 0.949 \)).

**Discussion**

The most rapid format for performing ELISA is currently the competitive type, because the assay procedure requires only a single incubation with antibody. Competitive ELISA, however, has several disadvantages with regard to the preferred "sandwich" ELISAs: inferior sensitivity, nonlinear absorbance response as a function of analyte concentration, and inferior precision owing to between-well variance in protein adsorption.

The between-well variability was minimized by using a coating concentration of antigen that was about 100-fold lower than the well binding capacity (16), ensuring quantitative and reproducible adsorption of antigen. Substrate mixing (17), combined with optimized formulation of the TMB substrate, produced a sensitive log-linear assay response over at least a 10-fold range. This compensated for the lower coating concentration and at the same time eliminated the imprecision in calculating unknown antigen values from the standard curve, because these values were determined by simple linear regression analysis. TMB was found to have many advantages over o-phenylenediamine as a peroxidase substrate: it is threefold more sensitive (18), it is noncarcinogenic (19), there is no need for the use of hazardous concentrated sulfuric acid, and it is more stable to autoxidation under room temperature storage.

We demonstrate that a microplate reader can be used in the correction of HUA by enzymatic determination of urinary creatinine, obtaining precision and accuracy comparable with that obtained by an automated analyzer. The enzymatic microplate creatinine assay was also well correlated with an automated kinetic Jaffé method when applied to plasma samples, although the values obtained by the enzymatic method were uniformly 20% to 30% lower than those obtained by the Jaffé method. This may be explained by the presence of chromogens in plasma (10) that interfere in the Jaffé method, whereas the enzymatic method is free from such interferences (11). This difference between assay methods can be corrected by using a commercially available creatinine calibration serum. Recommended storage conditions of no more than two weeks at 4 °C for the working solutions of the enzymatic kit are conservative, as we found no significant deterioration in assay response after at least a month of storage.

Normal control ranges for HUA, creatinine-corrected HUA, and albumin excretion rate correspond closely to those reported previously (6). Albumin excretion rate was found to exhibit the most narrow range and interindividual variation. Albumin measured in renal patients corresponded to total proteinuria, demonstrating the applicability of the HUA assay to proteinuria as well as microalbuminuria.

The use of random urine samples to identify patients whose albumin excretion rate is increased has been reported (20). In that study, use of the albumin/creatinine ratio reportedly was more sensitive and specific than determination of urinary albumin concentration alone. Simultaneous measurement of urinary albumin and creatinine may prove to be a useful method to screen patients for microalbuminuria.

1374 CLINICAL CHEMISTRY, Vol. 35, No. 7, 1989
We thank Boehringer Diagnostics for donating kits and calibration standards. This work was supported in part by grants from Dialysis Clinics, Inc. and the Kidney Foundation of Greater Cincinnati. Data management by the CLINFO system of the University of Cincinnati General Center for Clinical Research is supported by NIH grant MO1 RR00068.

References