Immunoturbidimetry of Urinary Albumin: Prevention of Adsorption of Albumin; Influence of Other Urinary Constituents

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I describe a simple, rapid immunoturbidimetric assay for low concentrations of albumin in urine (2 to 260 mg/L). However, in this assay, the human serum albumin (HSA) in the standards binds nonspecifically to the polystyrene or glass tubes. This nonspecific binding cannot be prevented by adding bovine serum albumin (BSA) to standards, because the anti-HSA antibody cross reacts with BSA. Adding Triton X-100 (1 mL/L) to standards effectively prevents this nonspecific binding of HSA from standards to both polystyrene and glass tubes. High concentrations of compounds found in urine from normal and diabetic subjects do not interfere with this assay if pH extremes can be avoided. The between-day CV is 4.8% at x = 18.8 mg/L and 2.0% at x = 183.1 mg/L. Measurements by this immunoturbidimetric method (y) correlate well with those obtained by a radioimmunoassay (x): y = 1.078x - 0.141 mg/L (n = 98, r = 0.984) and with those obtained by a radial immunodiffusion method (x'): y = 1.026x' - 0.117 mg/L (n = 98; r = 0.983). Urinary excretion of albumin by 25 healthy, nondiabetic subjects was ≤ 8 μg/min.

Additional Keyphrases: screening · kidney disease · diabetes · centrifugal analyzer · binding of albumin to glass, polystyrene

A slightly increased albumin excretion rate is considered predictive for the onset of chronic nephropathy and retinopathy (1, 2). Because this slightly increased rate may be decreased by strict control of glycaemia or concomitant hypertension, there is an increasing interest in screening and monitoring the excretion of albumin in diabetics.

Various methods are available for determining low concentrations of albumin in urine (3–7). Because immunoturbidimetry is a rapid and sensitive method available to the clinical laboratory, I have developed an immunoturbidimetric assay for quantifying low concentrations of urinary albumin with a centrifugal analyzer. Using this assay, I studied the problem of nonspecific binding of albumin from standards to both polystyrene and glass tubes. Only a few authors have reported taking measures to prevent this nonspecific binding (6–10). Some of them used bovine serum albumin (BSA) to prevent it (6–9), but in immunoturbidimetric assays this could interfere by cross reacting with the antibody used in the assay (11). Here I show that added Triton X-100 effectively prevents this nonspecific binding.

Because the composition of various urines can differ widely, I evaluated the effect of several urinary constituents on this assay by investigating a wide pH range and a variety of compounds encountered in normal and diabetic urines. Finally, I compared the results of this immunoturbidimetric assay with those obtained by a radial immunodiffusion assay and by those of a commercial radioimmunoassay.

Materials and Methods

Materials

Chemicals: Purified lyophilized human serum albumin ORHA 20/21 (HSA) was supplied by Behringwerke AG, Marburg, F.R.G.

Protein reference HOQ-3-TO1 was obtained from the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands.

Rabbit anti-human albumin antibodies (anti-HSA; prod. no. A001 and A119) were supplied by Dakopatts, Glostrup, Denmark.

All other chemicals, including Triton X-100 (prod. no. 12298), polyethylene glycol 6000 (PEG-6000; prod. no. 807491), and bovine serum albumin (BSA; prod. no. 12018) were obtained from Merck, Darmstadt, F.R.G.

Reagents: Phosphate-buffered saline (PBS, pH 7.4) was prepared in distilled water to contain 140 mmol of NaCl and 10 mmol of phosphate per liter.

Diluent was prepared by adding 15 mmol of NaNO₃ and 1 mL of Triton X-100 per liter of PBS.

The assay buffer consisted of phosphate buffer (5 mmol/L, pH 7.4) plus, per liter, 100 mmol of NaCl, 15 mmol of NaNO₃, and 45 g of PEG-6000.

To prepare the antiserum reagent, I diluted the A119 anti-HSA antibody 80-fold in assay buffer just before use.

BSA was dissolved in PBS, then serially diluted to give concentrations of 6, 13, 25, 50, 95, 190, 375, 750, 1500, and 3000 mg/L.

All reagents were stored at 4 °C.

Standards: A stock solution of HSA in PBS was prepared to give a concentration of about 1 g/L. To check the concentration of HSA, I measured the absorbance of the solution at 280 nm (HSA at 1 g/L should read 0.54 A). I diluted this solution fourfold with diluent to give a concentration of about 250 mg/L. This standard, which is stable at 4 °C, was diluted with diluent just before assay to concentrations of 2.0, 8.1, 32.5, 65.0, 130.0, and 260.0 mg/L for use in preparing the standard curve.

Controls: The HOO-3-TO1 reference serum was diluted with diluent to give controls with an HSA concentration of approximately 200 and 20 mg/L. These controls were kept frozen at −20 °C.

Samples

The collection of timed overnight urine specimens started with emptying the bladder at bedtime, discarding this specimen (time zero), and was stopped after the first morning urine was voided. These specimens were kept at room temperature during collection, then stored in glass tubes at −20 °C until assay. Reference values for albumin excretion rate were determined from data on urine specimens from 25 apparently healthy subjects working in this laboratory.

Assay Protocol

Allow urine samples to equilibrate at room temperature, then mix and centrifuge for 10 min at 500 × g. Use the clear
supernatant fluid for assay. To measure the concentration of urinary albumin by a kinetic immunoturbidimetric procedure (28), use the following settings for the "Multistat III" centrifugal analyzer (Instrumentation Laboratory, Ljuselstein, The Netherlands): 8 μL of standard or sample; 32 μL of distilled water; 120 μL of the antisera reagent; range of standard curve 2–260 mg/L; assay temperature 30 °C; wavelength 340 nm; time of first reading 3 s; time interval 30 s; no. of readings 12; calibration mode 2; and antigen excess detection at reading point 5.

Other Procedures and Studies

Adsorption of albumin: In the adsorption experiment I placed 500 μL each of two standards and five urines in 4-mL polystyrene (AFA-Polytek, Someren, The Netherlands) or glass tubes. Every 10 min (five times in all) I transferred the complete contents of each tube to another series of tubes. I then compared the delta absorbance at 340 nm of the contents of the fifth series of tubes with that of the solutions in the original tubes.

Interference study: To evaluate the effects of normal and diabetic urinary constituents, I tested the following solutions: NaCl, KCl, NH₄Cl, NaHCO₃, and Na₃PO₄, each at 250 mmol/L; CaCl₂, MgCl₂, and creatinine, each at 50 mmol/L; glucose and fructose, each at 500 mmol/L; urea, 1 mol/L; and 3-hydroxybutyric acid, 3-hydroxybutyric acid, and acetone, each at 600 mmol/L. At first I tested these substances dissolved in distilled water. In a second experiment I adjusted the pH of the solutions of 3-hydroxybutyric acid, 3-hydroxybutyric acid, and CaCl₂ to about 7.0 by adding NaOH or HCl, 1 mol/L.

In this interference study I diluted the set of standards with an equal volume of these respective solutions. Consequently, I doubled the sample volume in the assay, and decreased the volume of distilled water used by an equal amount before analyzing the standard curve.

Preparation of protein-free urines: I removed the protein from 18 urine samples by centrifugation with Centricon-10 microconcentrators (Amicon, Danvers, MA 01923). Some of these urines showed extreme values for pH, others for glucose, ketone bodies, or concentration of solutes.

Analytical-recovery study: To assess recovery, I diluted the set of standards 1:1 with each of the 18 protein-free urines. Consequently, I doubled the sample volume in the assay, and decreased the volume of distilled water used by an equal amount. The 18 "urinary" standard curves were compared with the standard curve ordinarily used.

Method comparison: The results of this new immunoturbidimetric assay for urinary albumin were compared with the results of a commercial radioimmunoassay (RIA) method (Pharmacia, Upplands, Sweden), performed according to the manufacturer's instructions, and with a radial immunodiffusion (RID) method, performed as described by Mancini et al. (9). For the RID I used agarose gels (10 g/L of PBS) containing 0.1 μL of the A001 anti-HSA antibody per cm². The results of the methods were compared by orthogonal regression analysis (13).

Results

Standard curve: I evaluated the composition of the assay buffer with regard to the concentration of phosphate, NaCl, and PEG-6000. Concentrations of phosphate ranging from 2.5 to 10 mmol/L, and concentrations of NaCl ranging from 100 to 150 mmol/L gave almost identical standard curves. High concentrations of PEG-6000 extended the useful range of the standard curve, but in the midrange the standard curve became less steep. A 45 g/L concentration of PEG-6000 in the assay buffer (concentration in the cuvet: 34 g/L) gave the steepest curve in the range from 2 to 260 mg/L. With an 80-fold dilution of the A119 anti-HSA antibody in this assay buffer there was an increasing turbidimetric signal till about 500 mg/L of HSA (Figure 1).

Adsorption of albumin: The results of the adsorption experiments (Figure 2) indicated that there was significant binding of albumin onto polystyrene tubes from both stan-
standards and urines; in contrast, glass tubes adsorbed albumin substantially only from standards.

**Effect of addition of BSA**: The authors who used addition of the carrier protein BSA to prevent nonspecific binding of HSA from standards applied a wide variety of concentrations of BSA. In order to determine the minimal concentration necessary for effective prevention of this nonspecific binding, I investigated a wide range of concentrations of BSA. However, BSA appeared to cross react in this immunoturbidimetric system (Figure 3a). Both the level and the course of the standard curve was influenced by the concentration of BSA (Figure 3b). This cross reactivity of the A119 anti-HSA antibody was confirmed by double immunodiffusion and immunoelectrophoresis. The A001 anti-HSA antibody also showed this cross reactivity. Therefore the capacity of BSA to prevent nonspecific binding of HSA was not studied further.

**Effect of addition of Triton X-100**: Nonspecific binding of HSA from standards could also be prevented by adding a surfactant. I evaluated the effect of different concentrations of Triton X-100. A 1 mL/L concentration of Triton X-100 completely prevented nonspecific binding of HSA from standards; lower concentrations did not, and concentrations >1 mL/L had no additional effects. In another adsorption experiment, no binding of HSA to polystyrene tubes and glass tubes was found (Figure 2).

Three experiments were performed to study the effect of Triton X-100 on the results for 20 patients’ urines. First, a standard curve was prepared without use of added Triton X-100 and the urines were analyzed (a). Secondly, a standard curve was prepared with addition of Triton X-100 and again the urines were analyzed (b). Finally, Triton X-100 was added to the urines before analysis with the standard curve of b (c). Comparison of the results showed significantly lower results for the urines if Triton X-100 was added to the standard curve. However, additional Triton X-100 in the urines did not again significantly alter the results: a: ± SD = 58.2 ± 60.8 mg/L; b: ± SD = 43.6 ± 55.5 mg/L; c: ± SD = 46.2 ± 62.4 mg/L; difference a – b: P < 0.001; difference b – c: n.s.

**Linearity**: Sequential dilution of urine samples with high concentrations of HSA yielded nonlinear results if no Triton X-100 was included in the diluent. After Triton X-100 was added to the diluent, sequential dilutions of urine samples gave linear results.

**Reproducibility**: I determined the precision profile from the data for 27 consecutive standard curves (Figure 4). Both home-made controls also were very reproducible, with a between-day CV of 4.8% for the low concentration (± 18.8 mg/L; SD = 0.9 mg/L; n = 27) and of 1.9% for the high concentration (± 183.1 mg/L; SD = 3.5 mg/L; n = 26).

**Influence of pH**: The pH of normal urines can vary between 5 and 10. I evaluated the effect of pH on the standard curve of this immunoturbidimetric assay for uri-
nary albumin. A range of assay buffers was prepared by changing the ratio between Na₂HPO₄ and NaH₂PO₄, within a pH range from 6.4 to 8.7 for assay buffers there was no significant change in delta absorbance for the four highest standards. At the higher end of the pH range there were some minor effects, but only at concentrations of HSA above the highest standard. The CVs for the standards analyzed at different pH were in the same order of magnitude as the CVs for the usual standards (Figure 4).

Influence of urinary constituents: I evaluated the effect on the standard curve of some compounds found in normal and diabetic urines. The first experiment did show a tremendous effect of some components (3-hydroxybutyric acid, 3-oxobutyric acid, and CaCl₂). However, concentrated solutions of acids can alter the pH of the reagent mixture; therefore, I repeated the experiment, using pH-adjusted solutions. With these there was, except for a minor effect of Na₂PO₄, no effect on the two highest standards (Figure 5). The other standards were not investigated in the second experiment, because the lower standards did not show any effect in the first experiment. The CVs for the standards in these experiments did not differ from those for the normal standards, if only the results of the pH-adjusted solutions were used for calculation (130 mg/L standard: t ± SD = 92.9 ± 1.4 mA; CV = 1.5%; and 260 mg/L standard: t ± SD = 142.3 ± 2.8 mA; CV = 2.0%).

Analytical recovery: In this experiment, each of the 18 "urinary" standard curves were analyzed once, together with two normal standard curves. The "urinary" standard curves did not show large deviations from the normal standard curves, although the CVs for the standards of the curves diluted in protein-free urines are a little higher than those for the normal standards. These data, including the calculated recoveries, are shown in Table 1.

Reference interval for albumin excretion rate: This range, estimated from data on timed overnight specimens from 25 apparently healthy subjects, was 3.6 (SD 2.2) μg/min. The upper limit for the normal albumin/creatinine ratio was 0.90 g/mol for this same reference group.

Sample comparison: Ninety-eight urine samples were assayed by immunoturbidimetry, RIA, and RID. Results correlated well for albumin concentrations below 80 mg/L (higher concentrations could not be measured by RIA). The results by immunoturbidimetry (y) and RIA (x) were related by the regression line: y = 1.078x - 0.141 mg/L (r = 0.984, standard deviation of intercept 0.548, standard deviation of slope 0.020), assuming equal imprecision in both assays. Turbidimetry yielded significantly higher results than RIA as calculated by Student's paired t-test (mean difference: 1.35 mg/L; P < 0.001). The results by immunoturbidimetry (y) and RID (x') were related by the regression line: y = 1.026x' - 0.117 mg/L (r = 0.983, standard deviation of intercept 0.539, standard deviation of slope 0.019), assuming equal imprecision in both assays. Turbidimetric results did not significantly differ from RID results by Student's paired t-test (mean difference: 0.4 mg/L).

Discussion

The importance of monitoring the albumin excretion rate as a predictor for the onset of clinical nephropathy and retinopathy in diabetic patients is becoming more and more apparent (1, 2). Therefore, a rapid and simple quantitative method for the measurement of urinary albumin would be of great value. The immunoturbidimetric assay described here is applicable for this purpose in a routine laboratory setting, in contrast to techniques such as radioimmunoassay, enzyme immunoassay, and nephelometric assay, which require specific instruments.

Investigation of the problem of nonspecific binding of HSA from standards to polystyrene and glass tubes revealed that it can substantially alter results for urine samples. Because the standard curve is prepared by serial dilution of the highest standard in polystyrene or glass tubes, the turbidimetric signals at the lower end of the standard curve will be reduced by this nonspecific binding of HSA from standards, which results in an increase in the sample results; therefore, preventive measures seem necessary. A carrier protein such as BSA (6–9), added to standards (and diluent), is frequently used to prevent this nonspecific binding. However, a wide variety of concentrations of BSA has been used. In investigating the minimal concentration of BSA necessary to prevent nonspecific binding, it appeared that BSA could not be used in this immunoturbidimetric assay, because the anti-HSA antibody shows cross reactivity

Table 1. Reproducibility and Analytical Recovery for Standards Diluted With Each of the 18 Protein-Free Urines (Determined Once)

<table>
<thead>
<tr>
<th>HSA concn, mg/L</th>
<th>x ± SD, mg/L</th>
<th>CV, %</th>
<th>Mean recovery (and range), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1</td>
<td>8.5 ± 1.55</td>
<td>18.2</td>
<td>105 (75–145)</td>
</tr>
<tr>
<td>32.5</td>
<td>31.9 ± 1.52</td>
<td>4.7</td>
<td>98 (86–106)</td>
</tr>
<tr>
<td>65.0</td>
<td>66.0 ± 4.50</td>
<td>6.8</td>
<td>102 (85–112)</td>
</tr>
<tr>
<td>130.0</td>
<td>136.5 ± 4.06</td>
<td>2.9</td>
<td>105 (100–113)</td>
</tr>
<tr>
<td>260.0</td>
<td>275.0 ± 10.79</td>
<td>3.3</td>
<td>106 (99–115)</td>
</tr>
</tbody>
</table>
with BSA. This cross reactivity did change both the level and the course of the standard curve. Therefore BSA only can be used for the prevention of nonspecific binding of HSA from standards in systems where the anti-HSA antibody does not cross react with BSA.

Addition of a surfactant such as Tween 20 to the standards also can prevent nonspecific binding of HSA from standards (10, 14). Here, I show that Triton X-100 also is effective. Triton X-100 added to the standards significantly changed the results for urines. Fortunately, addition of Triton X-100 to urine samples could be avoided, for such addition did not significantly alter the results for urines.

A consequence of the prevention of nonspecific binding of HSA from standards is the slightly lower upper limit of 8.0 µg/min for the reference interval for the albumin excretion rate, which agrees well with previously reported values of 6.4 (15) and 8.0 µg/min (16). These authors also used preventive measures. Authors who did not use these preventive measures found higher upper limits for this reference interval: 10.0 (5), 12.4 (17), 12.8 (18), and 13.2 µg/min (19). My upper limit for the ratio of albumin to creatinine (0.9 g/mol) also is slightly lower than the 1.01 g/mol found by Harmoinen et al. (5), who did not deal with adsorption.

Finally, standard curves for this immunoturbidimetric assay for urinary albumin excretion are very reproducible (Figure 4), which implies that the same standard curve can be used for some time. Therefore, this method can easily be used in an outpatient laboratory department. Considering the good day-to-day reproducibility of this new method, I also conclude that patients with low albumin excretion can be reliably distinguished from those without.

References