Laser Immunonephelometry for Routine Quantification of Urinary Albumin Excretion
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We describe a laser-immunonephelometric method for quantifying urinary albumin excretion (UAE) in large numbers of samples. For a 150-μL sample incubated at room temperature for 45 min with 40 μL of antiserum specific for human serum albumin, the assay range for albumin was 0.34 to 43.0 mg/L. For samples analyzed undiluted and diluted 10-fold, the range of measurable albumin was from 0.34 to 430.0 mg/L. With an automated version of this method, one can assay 240 samples per hour. Intra- and interassay CVs were <6% and 9%, respectively. Measurements by this method (y) correlated well with those obtained by a RIA method (x): y = 1.00x + 0.163 mg/L (n = 233; r = 0.996). The day-to-day CV for UAE was determined for three consecutive determinations done on each of 60 controls according to the time of collection and in 212 diabetics according to the amount of 24-h UAE. For controls, UAE was 8.0 ± 8.1 mg/24 h (mean ± SD), CV 44 ± 23%. The CV was similar for diurnal (50 ± 28%) and overnight (58 ± 32%) collections from controls; and for diabetics with normal values for UAE: 35 ± 32%, with slight albuminuria (25–300 mg/24 h): 37 ± 28%, or with macroalbuminuria (>300 mg/24 h): 47 ± 42%.

Additional Keyphrases: albuminuria • diabetes mellitus • screening • kidney disease • variation, source of

Quantification of urinary albumin excretion (UAE) is used for early assessment of lesions of the glomerulus in various pathological conditions such as essential hypertension (1) or kidney rejection after renal transplantation (2). In diabetics, a population with a high risk of hypertension and renal disease, slight albuminuria (3)—i.e., an UAE above normal values, but too small to give a positive reading with test strips—is predictive of subsequent renal failure in insulin-dependent diabetics (4) and of an increased cardiovascular mortality in non-insulin-dependent diabetics (5). Some researchers consider it justifiable to screen for pathological UAE in all diabetics, and to assess the effectiveness of treatment by monitoring this variable (6). However, the pilot studies cited above involved few patients and UAE was measured by RIA (7, 8), which cannot be used conveniently on a large scale. Thus, a simple, reliable method for measuring UAE is needed that can be applied to a potentially large number of patients.

We describe here a method for quantifying UAE routinely by laser immunonephelometry. Its performance is described and compared with that of a previously published RIA for urinary human albumin (9). Because UAE may be affected by a high intra-individual variation in diabetic subjects (10), we also studied factors that cause variations in measured UAE.

Materials and Methods

Apparatus and Assay Procedure

Laser immunonephelometry quantifies light scattering caused by antigen–antibody complexes that are precipitated in a liquid phase (11). In our apparatus (Behring Laser-Nephelometer, Module I; Behringwerke, Marburg, F.R.G.), light scattering from a laser source (632.8 nm) is converted into a voltage reading. Two other modules are connected to the instrument: Module II, for sequential readings at a pre-programmed time interval, and Module III, for automated distribution of standard or sample and antiserum. We used an AMP 85 calculator (Hewlett-Packard) to directly calculate albumin concentrations.

A 150-μL sample (standard or urine) is mixed with 160 μL of buffer (see below) in a disposable plastic cuvette, then 40 μL of pure antiserum specific for human serum albumin (HSA) is added to start the reaction. After a programmed time of 45 min, the then-stable voltage is measured. The absorbance of a sample can be read every 15 s, so that, with this automated procedure, 240 readings are possible per hour.

Reagents

We obtained NaH₂PO₄·H₂O, Na₂HPO₄, and sodium azide from Merck, Darmstadt, F.R.G.; bovine serum albumin (BSA; Cohn Fraction V; no. A-9647) from Sigma Chemical Co., St. Louis, MO. The antiserum and the standard used are commercially available from Behringwerke. Rabbit antiserum (batch no. 153 912) was specific for HSA as checked by immunoelectrophoresis, with a titer of 1 g/L. The standard used (batch no. 067 626) contained 43 g of HSA per liter (107.5 int. units/mL, in comparison with the WHO International Reference Preparation of six human serum proteins for immunoassay (22). Purified HSA (Cohn Fraction V; no. A 1 408) was from Sigma Chemical Co., as were bilirubin, hemoglobin, IgG, transferrin, β₂-microglobulin, and kappa and lambda light chains, all of human origin. Urea was from Merck.

Assay buffer consisted of phosphate-buffered saline (phosphate, 40 mmol/L, pH 8.0). We included BSA, 0.5 g/L, to

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3 Nonstandard abbreviations: UAE, urinary albumin excretion; BSA, HSA, bovine and human serum albumin. The terms "microalbuminuria" and "macroalbuminuria" are used by some clinicians to denote, respectively, slightly (> 25–300 mg/24 h) or grossly increased (>300 mg/24 h) UAE.

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avoid adsorption of small amounts of HSA onto the reaction cuvette (see below), and 1 g of sodium azide per liter as preservative. Stored at 4 °C, this buffer could be used for to three weeks with no interference in the assay procedure.

Assay Evaluation

Optimization studies: We evaluated incubation intervals of 15 to 90 min. Most experiments were conducted at room temperature (20–22 °C), but six standard curves were prepared from data on assays done at 30 °C.

The standard solution was diluted sequentially eight times (from 1000-fold to 128 000-fold) to give HSA concentrations of 43.0, 21.5, 10.76, 5.37, 2.69, 1.34, 0.67, and 0.34 mg/L. For reagent blanks we used 150 µL of buffer instead of standard.

To determine whether sample composition affected the reaction, we varied the pH of the phosphate buffer from 4.0 to 9.0; we also prepared samples in phosphate buffer (pH 8.0) to which various amounts of urea (0 to 8 mol/L) had been added, and we assessed the effect of adding other components to the pH 8.0 phosphate buffer: hemoglobin (100 mg/L), bilirubin (50 mg/L), IgG (10 g/L), transferrin (1 g/L), β2-microglobulin (1 g/L), and kappa or lambda light chains (1 g each per liter).

Urine samples were centrifuged at 1500 × g for 10 min, then assayed, either undiluted or diluted 10-fold. According to the range in which urinary HSA concentration could be measured in any given sample, we chose a dilution range of 0.34 to 430 µg/L. To test the nonspecific blank value of urine samples, we processed 20 urine samples as described above and compared results with those for the same samples to which 40 µL of buffer had been added instead of 40 µL of antiserum.

Analytical recovery and linearity: Increasing amounts of purified HSA were added to a urine sample containing <1 mg of HSA per liter (as determined by RIA), to give concentrations up to 64 mg/L. Conversely, we diluted one sample with a high HSA concentration (62.0 mg/L by RIA) 10- to 120-fold. These were all then assayed.

Intra- and inter- assay variation: To determine intra-assay variation, we measured three urine samples—one low, one medium, and one high concentration (see below)—20 times during the same day. For interassay variation, we measured 45 urine samples—15 low, 15 medium, and 15 high concentrations—once daily for five consecutive days.

Comparison of laser immunonephelometry and RIA methods: The RIA method we used for comparison was published recently (9) and derived from the technique described by Miles et al. (8). It allows routine measurements of urinary HSA between 1 and 100 mg/L. Briefly, the procedure is as follows. The sample volume is 5 µL and a 3-h incubation at 4 °C is required. We made some slight technical modifications: after precipitation of the antibodies with polyethylene glycol (M, 6000), we drained the supernate and counted the radioactivity in the precipitate. Thus, HSA concentrations were calculated from B/B0, the percentage of tracer bound to antibody for each sample, as compared with the zero point, after subtraction of nonspecific binding. The initial antiserum dilution was 9000-fold and tracer activity per tube was about 10 000 counts/min (specific activity 60 Ci/g). As little as 0.1 mg of HSA per liter could be detected if we used an initial antiserum dilution of 36 000-fold. The results presented here were for samples, diluted fourfold or more when necessary, assayed in duplicate. Results were considered valid if the results for the two dilutions paralleled the standard curve with a variation of <10% for each sample.

Subjects

We studied 60 healthy controls—28 men, 32 women; ages 40 (SD 13 years, range 23–60 years) and weighing 65 (range ±18) kg—with three different urine-specimen-collection procedures: 24-h, diurnal (from 08:00 to 23:00), and overnight (from 23:00 to 08:00). To calculate the CV of the UAE, we asked each subject to make each type of urine collection three consecutive times. Moderate physical activity was authorized during the study period. Results were compared with the mean for three successive 24-h urine collections for 212 diabetic patients (149 insulin-dependent; 63 non-insulin-dependent) of comparable sex distribution (111 men; 101 women), age (48 ±14 years), and weight (63 ±20 kg). The patients were classified according to the stage of their diabetic retinopathy (assessed by ophthalmologic examination and fluorescein angiogram): stage 0 (no retinopathy); stage 1 (background retinopathy); stage 2 (retinopathy plus maculopathy); stage 3 (proliferative retinopathy) (13).

The urine specimens were kept at room temperature during collection, then stored at −20 °C until assay. Urine specimens were divided into three groups according to their HSA concentrations (as measured by RIA): low (<5 mg/L), medium (5–20 mg/L), and high concentrations (>20 mg/L).

Statistical Analysis

Results are presented as mean values ± 1 SD. We used the least-squares method for comparison of laser immunonephelometry and RIA methods, and Student’s t-test to compare the effects of the time of urine collection and the subjects’ characteristics on UAE values and CV.

Assay Evaluation

Voltage readings at room temperature for HSA concentrations of 43.0 to 0.34 µg/L are shown in Figure 1 for incubation intervals ranging from 5 to 90 min (10 determinations for each point). The voltage was stable at 30 and 45 min. Voltage variability was high at 60 min for 43.0 mg/L (11.1 ±1.1 V), and voltage declined for longer incubation times, suggesting a prozone phenomenon (14). We therefore chose to use a 45-min incubation thereafter. Data on voltage readings for six standard curves, with incubation at 30 °C,
were indistinguishable from those read at room temperature (20–22 °C): 11.20 ± 0.31 V for 43.0 mg of HSA per liter; 8.35 ± 0.21 V for 21.5 mg/L; 5.85 ± 0.03 V for 10.76 mg/L; 3.64 ± 0.02 V for 5.37 mg/L; 2.17 ± 0.04 V for 2.69 mg/L; 1.23 ± 0.02 V for 1.34 mg/L; 0.78 ± 0.02 V for 0.87 mg/L; and 0.40 ± 0.01 V for 0.34 mg/L.

We tested the detection range of the method by incubating HSA concentrations from 0.1 to 500 mg/L for 45 min at room temperature (Figure 2). The voltage increased significantly for 0.2 mg of HSA per liter as compared with the zero value (0.31 ± 0.01 V vs 0.25 ± 0.01 V; 10 determinations; p < 0.001). Voltage was maximal for 50 mg of HSA per liter, then decreased abruptly. When BSA was omitted from the buffer, the sensitivity of the method was reduced to 5 mg/L. At this concentration, voltage was significantly lower than with BSA (1.72 ± 0.05 V; p < 0.001).

We determined the CVs for each point of the standard curve from voltage readings on five consecutive days: 43 mg/L, CV = 3.1%; 21.5 mg/L, CV = 1.7%; 10.76 mg/L, CV = 2.7%; 5.37 mg/L, CV = 2.8%; 2.69 mg/L, CV = 3.3%; 1.34 mg/L, CV = 3.2%; 0.87 mg/L, CV = 2.1%; and 0.34 mg/L, CV = 4.1%.

**Effect of Sample Composition**

When we modified the pH used for the standard curve from 4.0 to 9.0, the voltage readings did not change significantly, nor did urea concentrations from 0 to 4 mol/L alter voltage readings, but 8 mol of urea per liter gave decreases in voltage of 80% for 21.5 mg of HSA per liter and of 35% for 43 mg/L. We found no interference by the following other possible components of urine: bilirubin, hemoglobin, IgG, transferrin, β2-microglobulin, or kappa and lambda light chains.

**Evaluation of the nonspecific blank values of urine samples.** For 20 urine samples we measured a blank value of 0.19 ± 0.07 V, which is negligible as compared with the voltage corresponding to the initial concentration (0.34 mg/L) of the standard curve (p < 0.001). HSA concentrations were almost identical with and without subtraction of the urine blanks: 3.98 ± 0.05 mg/L vs 3.82 ± 2.95 mg/L.

**Recovery and linearity.** We added increasing amounts of HSA, to give concentrations from 2.0 to 64.0 mg/L, to portions of one urine sample and, on assaying, obtained values equal to 101% ± 3% of the expected ones. For sequential dilutions of a 62 mg/L urine sample, measured values were 98% ± 4% of the expected.

**Reproducibility experiments.** The intra-assay CVs for three urine samples containing 1.7, 9.7, and 54.2 mg of HSA per liter were respectively 5.11, 5.23, and 2.93%. Median inter-assay CV was 2% (range 1–5%) for 15 low values (range 1.5–4.0 mg/L), 3% (range 1–9%) for 15 medium values (range 5.0–20.0 mg/L), and 4% (range 1–7%) for 15 high values (range 21.0–62.0 mg/L).

**Comparison of RIA and laser-immunonephelometry methods.** Analysis of 233 urine samples (HSA concentrations 0.5 to 125.0 mg/L by the RIA method) gave good correlation between the RIA method (x) and the laser-immunonephelometry method (y): y = 1.00x + 0.163; r = 0.956; p < 0.001 (Figure 3).

Correlation was comparable at low and medium HSA concentrations (from 0.5 to 20.0 mg/L by RIA): y = 1.01x + 0.07 (r = 0.975; n = 178; p < 0.001) and for the high HSA concentrations (from 20.3 to 125.0 mg/L by RIA): y = 1.01x + 0.04 (r = 0.987; n = 54; p < 0.001).

**UAE in controls and in diabetic patients.** Table 1 gives the mean UAE for 60 healthy subjects according to the type of urine collection. Diurnal UAE was higher than overnight UAE (p < 0.05). The mean day-to-day CV for the 24-h UAE was significantly lower than that of overnight UAE (p < 0.01). Values were similar for men and for women, nor did we find any age-related effect. In the diabetics, the mean day-to-day CV for UAE was not affected by the amount of UAE (Table 2). The distribution of the mean 24-h UAE is shown in Figure 4 for healthy subjects and diabetic patients. In both populations, UAE was skew-distributed, being <5 mg/24 h for 53% of the healthy subjects. Only one of the 60 healthy controls had a mean UAE as high as 27 mg/24 h. Of the 212 diabetics, 30 (14%) had one or more values for UAE discordant with the mean of the three measurements.

In diabetics with no or only background retinopathy, the median UAES were respectively 11.2 (range 2.1–35.1) and 33.7 (range 2.2–381.6) mg/24 h. They were significantly higher in patients with a maculopathy (median 437 mg/24 h, range 4.1–1890; p < 0.005) or proliferative retinopathy (median 1242 mg/24 h; range 4.2–7400; p < 0.001).

![Fig. 2. High-dose "hook" effect for assay of HSA with 45-min incubation at room temperature. Shown are the mean and SD for 10 determinations each.](image-url)

**Table 1.** UAE and Type of Urine Collection in 60 Healthy Subjects (Three Urine Collections from Each Subject for Each Type of Collection)

<table>
<thead>
<tr>
<th>Urinary albumin excretion</th>
<th>Type of collection*</th>
<th>Mean ± SD*</th>
<th>Upper normal value (mean ± 2 SD)</th>
<th>CV, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diurnal, µg/min</td>
<td>6.6 ± 7.7</td>
<td>22</td>
<td>50 (28)</td>
<td></td>
</tr>
<tr>
<td>Overnight, µg/min</td>
<td>4.2 ± 4.1</td>
<td>12.4</td>
<td>58 (32)</td>
<td></td>
</tr>
<tr>
<td>24-h, mg/24-h</td>
<td>8.0 ± 6.1</td>
<td>24.2</td>
<td>44 (23)</td>
<td></td>
</tr>
</tbody>
</table>

*Diurnal: 08:00–23:00; overnight: 23:00–08:00. **Mean of three collections per category per subject. *Mean (and SD). p < 0.05 between diurnal and overnight. *p < 0.01 between overnight and 24-h urine collections.

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Table 2. Mean Day-to-Day CV According to Extent of Urinary Albumin Excretion

<table>
<thead>
<tr>
<th>UAE</th>
<th>No. patients</th>
<th>UAE, mg/24-h</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, &lt;24 mg/24-h</td>
<td>135</td>
<td>8.6 ± 5.0</td>
<td>35 ± 32</td>
</tr>
<tr>
<td>Slight albuminuria, 25-300 mg/24-h</td>
<td>63</td>
<td>84.9 ± 65.0</td>
<td>37 ± 28</td>
</tr>
<tr>
<td>Macroalbuminuria, &gt;300 mg/24-h</td>
<td>14</td>
<td>458.5 ± 218.2</td>
<td>47 ± 42</td>
</tr>
</tbody>
</table>

*Results for three consecutive 24-h collections from 212 diabetic patients.

Fig. 4. Distribution of UAE values in the healthy subjects (black bars) and in the diabetic patients (hatched bars)

Discussion

We show here that UAE can be quantified routinely by the method we describe. Many patients can be screened rapidly for pathological UAE with this relatively simple technique. The sensitivity and reproducibility of the method are such that accurate measurements of UAE can be made in normal subjects as well as in patients with glomerular diseases. Antigen–antibody complex is simply and reliably measured with this laser-nephelometer apparatus, because the light scattered by the complex is directly converted into a voltage reading. Blank values from this reaction are negligible, so albumin concentration can be obtained directly from light scattering, thus avoiding the calculation of a percentage of relative light scattering. This differs from another laser immunonephelometry system reported recently (13).

We checked conditions that might interfere with the reaction: buffer composition, pH and urea concentrations of the tested samples, the environmental temperature, and the duration of incubation. These dilution and recovery studies and reproducibility experiments all gave satisfactory results. The present method and the radioimmunoassay method gave comparable results. No interference was noted by compounds that may be found in urine: hemoglobin, bilirubin, or various proteins.

Although the RIA method used in this study was especially designed for rapid screening for pathological albumin excretion within a wide range of values (1–100 mg/L), it appeared more time-consuming than the laser-immunonephelometry method when a large number of urine samples are to be assayed. Moreover, the use of radiiodinated albumin for RIA is avoided with laser immunonephelometry. Lastly, the sensitivity of the two methods is similar.

Methods other than RIA have been developed in the past years for detecting pathological UAE. Some are semiquantitative (16, 17). Others have relatively poor sensitivity (18, 19); although this is not the case for latex (20, 21) or RIA (19) methods, they are inconvenient for quantification of UAE in large groups of patients.

Several new insights have appeared recently into the significance of microalbuminuria in diabetics (4, 5). From these pilot studies conducted on a relatively small number of patients, it may be inferred that pathological albumin excretion should be detected routinely in the diabetic patients. Hitherto, this could only be monitored in a limited number of care units devoted to diabetics. The present method now provides a tool suitable for use in routine detection. The relatively high variability of UAE may lead to false detection of microalbuminuria. The mean UAE is higher during the day than during the night, a fact which can be explained by the increase in UAE with physical exercise (23). Therefore the time of urine collection must be stated, with the appropriate upper value for normal UAE. However, the day-to-day CV for UAE appeared to be independent of both the time of collection and the degree of UAE. Repeat measurements of UAE seem necessary if microalbuminuria is to be confirmed. If this precaution is observed, the method described in this paper should encourage large-scale studies on UAE in diabetics and other populations that are prone to kidney disease, and assessment of the efficacy of new forms of therapy as reflected by this variable.

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