Method for Measuring the Concentration of Urinary Proteins According to Their Molecular Size Category

Amadeo J. Pesce, A. Hsu, C. Kornhauser, K. Sethl, B. S. Ool, and V. E. Pollak

We combined the use of a concentrating device (Minicon) and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to semi-quantitate the concentration of (a) the collective low-molecular-weight proteins and (b) of albumin excreted in the urine of patients after renal transplantation. Analytical recovery of many serum proteins from samples concentrated 100-fold in the Minicon apparatus was about 70%. It was possible to examine many urine samples by polyacrylamide gel electrophoresis after concentration with this device. The reproducibility (CV) of the technique was on the order of 20% when albumin and low-molecular-weight protein were in about equal concentration. The method was adequate to differentiate glomerular and tubular proteinuria, because in glomerular proteinuria the ratio of albumin to low-molecular-weight proteins is about 20/1, whereas in tubular proteinuria the ratio is about 1/1.

Additional Keyphrases: glomerular vs. tubular proteinuria • β₂-microglobulin • concentration technique • screening proteinurias

In making diagnostic use of the urinary proteins in disease, it is important to differentiate these proteins on the basis of their molecular size (1–5). Most proteins excreted in the urine are from serum (6); albumin, transferrin, and γ-globulin predominate, but there are some serum proteins with molecular weight of less than 70 000 (7, 8). Proteinuria of glomerular origin can be distinguished from that associated with tubular disease by the molecular size of the proteins excreted in the urine (1, 3). Until recently, the most rigorous techniques for examining urinary proteins have been limited to Sephadex-gel column chromatography and radial immunodiffusion (4, 8); both of these are time consuming, and therefore few urines can be examined. Methods used for the biochemical assessment of tubular proteinuria for large numbers of samples include zone-electrophoretic methods on paper, starch gel, and polyacrylamide gel (9); in none of these is mobility of a protein proportional to its molecular size. Thus conflicting data can be obtained with similar patient populations (10, 11). A method is needed for study of many urine protein samples that is also suitable for examining a broad spectrum of proteins in terms of molecular size.

In this paper we describe a method for concentrating many samples of urinary protein by using the “Minicon” concentration device. We also report slight modifications of the method for the semi-quantitative measurement of urinary proteins based on molecular size, by using polyacrylamide gel electrophoresis in the presence of detergent (12).

Methods

Apparatus

Minicon concentrators. Type B-15 “Minicon” concentrators were obtained from the Amicon Corp., Lexington, Mass. 02173. These were all from lot No. C0028A.

Acrylamide gel system. We used the Model R-113 acrylamide gel system (Beckman Instruments, Inc., Fullerton, Calif. 92634).

Scanning densitometer. We used the “Quick Scan Electrophoresis Densitometer” (Helena Laboratories, Beaumont, Tex. 77704).

Reagents

Acrylamide and bisacrylamide were purchased from Eastman Organic Chemicals, Rochester, N. Y. 14650, and used without further purification; sodium dodecyl sulfate from Sigma Chemical Co., St. Louis, Mo. 63178; lysozyme from Worthington Biochemical Corp., Freehold, N. J. 07728; human albumin from the Research Division of Miles Laboratories, Kankakee, Ill. 60901; and Coomassie Brilliant Blue (sodium azolene) from Schwarz/Mann Corp., Orangeburg, N. Y. 10962. All other chemicals were reagent grade.

Working Solutions

SDS/Tris/glycine pH 8.4 electrophoresis buffer.² Dissolve 21.8 g of ammonia-free glycine, 4.5 g of

² Nonstandard abbreviations used: SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane [more properly, tri(hydroxymethyl)methylamine]; PAGE, polyacrylamide-gel electrophoresis; and ID, immunodiffusion.

Division of Nephrology, Department of Medicine, University of Cincinnati Medical Center, Cincinnati, Ohio 45267.

¹ Present address: Escuela de Medicina de León, 20 de Enero No. 915, León, Guanajuato, Mexico.

² Received May 26, 1976; accepted Mar. 5, 1976.
tris(hydroxymethyl)aminomethane, and 1 g of sodium dodecyl sulfate in 80 ml of water and dilute to 1 liter.

Coomassie Blue staining solution. Add 1.25 g of Coomassie Blue to a mixture of 454 ml of methanol, 454 ml of water, and 46 ml of glacial acetic acid.

Destaining solution. Mix 650 ml of water, 250 ml of methanol, and 100 ml of glacial acetic acid.

Acetic acid solution. Add 20 ml of glacial acetic acid to 1 liter of water.

SDS solution. Dissolve 100 mg of sodium dodecyl sulfate in 10 ml of water.

Bovine serum albumin standards. Dissolve 300 mg of bovine plasma albumin (Armour Laboratories, Kankakee, Ill.) in 150 ml of water. Separate into 10 aliquots and freeze. For use, add 0.1, 0.2, 0.3, 0.5, 1.0, and 1.5 ml of this to a series of tubes and dilute to 5 ml with water.

Urine samples

Collect 24-h urine specimens with thymol preservative. Refrigerate, centrifuge, freeze, thaw, and re-centrifuge all urines to precipitate most of the phosphates and urates.

Procedures

Biuret test procedure. Add 5 ml of trichloroacetic acid solution (200 g/liter) to three 5-ml samples from each patient and to the standards. Mix and allow the solutions to stand for 20 min. Centrifuge for 15 min at 1800 × g in a clinical centrifuge. Decant the supernatant solutions. Dissolve the precipitate in 1 ml of 1 mol/liter NaOH. Add 2 ml of the usual biuret reagent to two of the three samples and 2 ml of NaOH to the third. Mix and allow the solutions to stand for 20 min. Read the absorbance at 540 nm. Subtract the absorbance of the sample redissolved in 1 mol/liter NaOH from those with biuret reagent. Read the value of the protein content from a curve of the absorbance of the albumin solutions vs. amount of albumin.

Protein Electrophoresis on Polyacrylamide Gel

The procedure used is similar to that described previously (12) but the staining procedure is changed as described below.

Sample preparation. Good results are obtained when the protein content of the samples is about 10 g/liter. The results of the biuret test on urine were used in calculating the sample concentration. All samples were concentrated by adding 10 ml to each well of the Minicon apparatus and allowing the sample to concentrate to the 25× mark (50-fold concentration). The protein concentration was adjusted to 10 g/liter by dilution with the Tris/glycine/detergent buffer. If the protein solution was so dilute that this concentration could not be attained, we made several applications of the sample from the Beckman dispenser.

Samples were prepared for application by mixing 50 μl of the concentrated urine protein, 5 μl of the SDS solution and one crystal of sucrose (to increase sample density). The samples were incubated for 15 min before being added to the gel. For comparison, a sample of normal serum and a mixture, standardized by weight, of 5 mg of lysozyme, 10 mg of human γ-globulin, and 8 mg of human albumin per milliliter were included in each gel.

Procedure. Dissolve 0.125 g of N,N'-methylenedibisacrylamide and 2.375 g of acrylamide in 35 ml of the Tris/glycine buffer and then filter through Reeve Angel filter paper No. 802. To prepare the main bottom gel, dissolve 20 mg of ammonium persulfate in 22 ml of the filtered acrylamide solution. Add 33 μl of N,N,N',N'-tetramethylethylenediamine, quickly mix thoroughly and pour into the mold, place the sample well mold into its proper place, and allow the gel to polymerize for 45 min. Prepare the top gel mixture by dissolving 10 mg of ammonium persulfate in 11 ml of the acrylamide filtrate, add 17 μl of the N,N,N',N'-tetramethylethylenediamine mixture, and pour into a gel mold until full. Immediately apply the samples with the applicator into the preformed wells. Each delivery of the applications is about 5 μl. Up to five applications may be made without decreasing the resolution. After polymerizing for 45 min, start the cooling water, insert the wicks, and place the unit into the electrophoresis chamber. Apply a voltage of 400 V (about 40 mA) for 45 min. Remove the gel, place in the staining solution, and incubate for 45 min at 55 °C.

Rinse off the stain several times with destaining solution and place in the Beckman destaining apparatus for 12 min with the dilute acetic acid solution. Continue removing the excess dye by incubating the gel in the destaining solution at 55 °C for several hours and then soaking for several days at room temperature.

Dry the gels between two membranes under tension, as recommended by the manufacturer.

Scan the gels with the Helena Densitometer, using the wide slit opening and the long-scan and fast-speed settings. Adjust the albumin value for each sample to about 80 arbitrary intensity units. Reset this to a smaller value when the low-molecular-weight material has an intensity greater than 90 units. Obtain the amount of albumin, γ-globulin, and low-molecular-weight protein from the integration-pen markings.

Recovery of Proteins from the Minicon Concentrator

Preliminary studies were done in which normal human serum was diluted and concentrated with the Minicon Concentrator. Estimates of quantitative recovery were limited by the difficulties in establishing the amount of protein that had adsorbed to the apparatus or leaked into the absorbent pads. To overcome this problem, radiolabeled albumin was added to the diluted serum.

Rat serum albumin was labeled by the method of Gaizutis et al. (13), except that no carrier bovine serum albumin was added to the iodinated albumin before it was applied to the Sephadex G-25 column. One milliliter of human serum was diluted with 99 milliliters of phosphate-buffered saline, and 20 μl of labeled rat al-
Table 1. Recovery of \(^{125}\)I-labeled Rat Serum Albumin Added to Serum Diluted 100-fold in Phosphate-Buffered Saline and Concentrated 100-fold with the Minicon B-15 Membrane\(^a\)

<table>
<thead>
<tr>
<th>Actual measurements</th>
<th>Protein-bound, corrected for 4.71% free (^{125})I</th>
<th>Corrected for 1.22% loss in initial transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(94.3 \pm 3.1)</td>
<td>(94.3)</td>
</tr>
<tr>
<td></td>
<td>(1.2 \pm 0.2)</td>
<td>(1.2)</td>
</tr>
<tr>
<td></td>
<td>(12.1 \pm 2.1)</td>
<td>(7.8)</td>
</tr>
<tr>
<td>% Counts retained by the membrane</td>
<td>Adsorbed to membrane (4)</td>
<td>Extracted by washing (5)</td>
</tr>
<tr>
<td></td>
<td>(8.9 \pm 2.6)</td>
<td>(9.3)</td>
</tr>
<tr>
<td>% Protein retained</td>
<td>(91.4)</td>
<td>(85.2)</td>
</tr>
</tbody>
</table>

\(^a\) Results are expressed as the mean percentage recovery, ±SD, for eight experiments.

\(^b\) % of total counts recovered from all sources.

\(^c\) % counts lost in initial transfer into the apparatus.

\(^d\) Counts lost by transfer through the membrane into the absorbent.

bumin was added. Ten milliliters of this mixture of diluted human serum and labeled rat albumin was added to each of the eight wells of the Minicon apparatus, and the solutions allowed to concentrate to the 50X mark on the apparatus. The net concentration was 100-fold. The concentrated sample was removed with a Pasteur pipet. Each sample well of the apparatus was then flushed with 2 milliliters of phosphate-buffered saline, labeled as the wash solution. The apparatus was then split with a hammer and screw driver, and the pad, sponge, and membrane for each section separated and placed into counting vials. A significant amount of radioactive material was also found on the transfer pipet; this value was recorded. There was a negligible amount of labeled material on the clear plastic. The amount of material found on the sponge was very small and, for purposes of reporting, was combined with that found in the absorbent pad.

Radial Immunodiffusion

Use the procedure of Mancini (14), with "Behring M Partigen Immunodiffusion" plates (Behring Diagnostics, Somerville, N. J. 08816). Add 5 μl of sample, allow the reaction to equilibrate for 48 h.

\(\beta_2\) Microglobulin Assay

Use the "Phadebas \(\beta_2\)-microtest" (Pharmacia Diagnostics, Piscataway, N. J. 08854).

Results

Table 1 summarizes the results of experiments in which \(^{125}\)I-labeled rat serum albumin was added to normal serum diluted 100-fold and concentrated 100-fold with the Minicon B-15 membrane. In all, 94.3% of the total counts added were accounted for from all sources. In the second and third lines of Table 1, corrections were made for the 4.7% free \(^{125}\)I in the labeled rat serum albumin added, and for the 1.2% lost in the pipet during the initial transfer into the concentrating apparatus.

The amount of material recovered in the concentrated solution when the usual pipetting procedure was used was about 63%. An additional 9% was recovered from the pipet, and additional material was extracted by washing the apparatus with 2 milliliters of buffer. The total recovery by this procedure (Table 1, columns 5, 6, and 7) was about 77%. However, the amount readily obtained from the concentrating apparatus (columns 5 and 7) was only 67% of the starting material. Thus for normal operating procedures, about 33% of the starting material was lost, 9% because of adsorption to the
membrane or passage into the absorbent and the rest during transfer pipetting. The latter was not extractable without a wash solution. The data on line 1 of Table 1 indicate that the apparatus gave good reproducibility from sample to sample.

In a second experiment, four different samples of human serum from normal individuals were added to $^{125}$I-labeled rat albumin; each sample was then diluted 50-fold in phosphate-buffered saline and concentrated in the Minicon B-15 concentrator. The amounts of nine human serum proteins were estimated by radial immunodiffusion in the native serum and in the diluted and concentrated samples (Table 2). Recovery of added $^{125}$I-labeled rat albumin averaged 78.6%; that of the nine native serum proteins varied from 63.8 to 85.3%; thus there was no differential loss of any protein.

Reproducibility of the Polyacrylamide Gel Sodium Dodecyl Sulfate Technique on Urine Samples

A single urine sample was placed in the gel, as well as two- and fourfold dilutions of this sample. In all, eight replicates at three different concentrations each were quantitated on three different gels. Mean values for albumin and low-molecular-weight proteins were respectively 73 and 27%, i.e., an albumin/low-molecular-weight ratio of 2.7/1. The coefficient of variation for the estimate of albumin was 9%; that for low-molecular-weight protein was 26%.

The reproducibility was tested similarly on the urine of a single patient with glomerular disease. Mean values for albumin and low molecular-weight proteins were 92
and 8%, respectively—i.e., a ratio of 11.5:1. The CV for the estimate of albumin was 4.6%; that for low-molecular-weight protein was 53%.

Serial Observations on Urine

Figure 1 shows a representative series of gels in which the proteinuria in a single patient after renal transplantation is assessed. In all urines, albumin and low-molecular-weight proteins predominated. Figure 2 shows the results of studies over a period of 134 days in this patient.

Measurement of Protein Concentration by SDS/Polyacrylamide-Gel Electrophoresis and by Quantitative Immunoochemical Techniques

To evaluate the results obtained with the SDS/polyacrylamide gel electrophoretic method (SDS-PAGE) in urines from patients with tubular disorders, we also analyzed 51 urine samples studied by this method for albumin content, by radial immunodiffusion (ID). The results are shown in Figure 3. The equation describing this relationship was:

\[ \text{Albumin (SDS-PAGE)} = 1.54 \text{ albumin (ID)} - 0.001 \]

A similar relationship was observed whether the 19 paired specimens with the low values for albumin (≤30 mg/liter) were included in or excluded from the regression analysis.

The \( \beta_2 \)-microglobulin content of these 63 urine samples was measured by radioimmunoassay, and the results compared with the estimate of low-molecular-weight protein content measured by SDS/polyacrylamide gel electrophoresis (Figure 4). Overall, the correlation between \( U_{\beta_2 \text{-microglobulin}} \) V and \( U_{LMW} \) V was poor. Nevertheless, both methods predicted equally well the increased amount of low-molecular-weight protein in urine from patients with tubular disease. The normal and upper limit of normal of \( U_{LMW} \) V is \( \approx 6-10 \) mg/24 h (8, 14) and of \( U_{\beta_2 \text{-microglobulin}} \) V is \( \approx 82 \) and 370 mg/24 h (15). Using these criteria for the upper limit of normal, the relative diagnostic value is summarized in Table 3. Concordant results were obtained in 50 of the 63 specimens, and discordant results in 13. Of interest is the observation that six of the seven urines with high \( \beta_2 \)-microglobulin excretion and normal low-molecular-weight protein excretion were from a single one of the eight patients studied; and that four of the six urines with normal \( \beta_2 \)-microglobulin content and high low-molecular-weight protein content were from another patient.

**Discussion**

**Use of the Minicon Concentrator**

Although some protein loss did occur, the Minicon Concentrator gave very reproducible results. Mechanical loss amounted to about 13%, and there definitely was some adsorption of protein to the membrane. Despite this loss, there was no selective loss of any serum protein measured. The convenience of the device and its reproducibility make it well suited for the study of a large number of samples.

---

**Table 3. Relation between Excretion of Low-Molecular-Weight Protein (\( U_{LMW} \) V) and of \( \beta_2 \)-Microglobulin (n = 63 Paired Urines)**

<table>
<thead>
<tr>
<th>( U_{LMW} ) V</th>
<th>Normal</th>
<th>Increased</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Increased</td>
<td>6</td>
<td>49</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** Urine albumin concentration in 51 urine samples from patients with renal tubular disorders, as measured by immunodiffusion (ID) and by sodium dodecyl sulfate/polyacrylamide-gel electrophoresis (SDS-PAGE).

*The interrupted lines denote the lower limits of accurate measurement by each technique, the open circles values below these limits. The solid line was calculated by regression analysis.*

---

**Fig. 4.** Urinary excretion rates of \( \beta_2 \)-microglobulin and low-molecular-weight protein compared.

*The 63 samples were from patients with renal tubular disorders. The interrupted lines denote the lower limits of accurate measurement by each technique, the open circles values below these limits.*
Semi-Quantitative Analysis of Proteins by Polyacrylamide Gel Electrophoresis

We indicated earlier (13) that the procedure was not accurately quantitative; the destaining method has been improved. There is less background and less variability, so that the gel can be scanned by using a densitometer with minimal background interference. Because there are now no standards for the many low-molecular-weight proteins excreted in urine, absolute quantitation of all low-molecular-weight proteins by this or any other method is not possible. Nor is it practical to make use of any method such as Sephadex-gel chromatography or quantitative immunoassay of many serum proteins in urine.

For these reasons, the semiquantitative SDS/polyacrylamide gel electrophoretic method can only be evaluated against specific methods for certain selected proteins. Increased β2-microglobulin excretion is considered to be an important marker for dysfunction of the renal tubules (16). The data in Table 3 indicate that, for the diagnostic purpose of detecting tubular dysfunction, the two methods did not differ. As there are many low-molecular-weight proteins in the urine, it is not surprising that some urines should show only a high β2-microglobulin excretion whereas a few others with an increased amount of low-molecular-weight protein did not show a high excretion of β2-microglobulin. The fact that most of these differences did not occur randomly but in urines from two particular patients suggests biological rather than technical factors.

Different results will be obtained when albumin is measured by different methods, particularly when one method is specific and the second measures albumin and other proteins within a certain range of molecular size. Qualitatively, analysis of tubular proteinuria by SDS/PAGE and column chromatography on Sephadex G-75 gave similar results (12). In the detailed analysis (at 280 nm) of the molecular size distribution of protein molecules in tubular proteinuria Dillard et al. reported (8): albumin, 30%; mol wt 60 000-40 000, 33%; mol wt ≤20 000, 27%; and mol wt >70 000, 9%. The Minicon B-15 concentrator, in contrast to the Amicon UM-10 membrane used in their study (8), has a nominal cut-off point of 15 000 mol wt and a significant proportion of proteins of mol wt <20 000 will have been excluded from analysis. As the albumin concentration using SDS/PAGE is estimated by the equation:

\[
\text{Albumin (SDS/PAGE)} = \frac{\text{total protein} - \text{low-molecular-weight protein}}{\text{mol wt}}
\]

the exclusion of up to 27% of the total protein (molecules of <20 000 mol wt) leads to a corresponding overestimate of the albumin content. Also γ-globulin and proteins of high molecular weight bind the Coomassie Blue so poorly that the small amounts (9%) present in urine were not estimated and were therefore ignored. This also leads to an overestimate of the amount of albumin. Taking these factors into consideration the following relationship would be anticipated:

\[
\text{Albumin (SDS/PAGE)} = 1.57 \text{ albumin (ID)}
\]

This figure is very close to that observed.

The observations reported herein show clearly that the SDS/PAGE technique for analyzing urine proteins is a valid method for the study of proteinuria associated with renal tubular dysfunction. Although the technique is not strictly quantitative and does not measure individual serum proteins in urine with precision, it does measure proteins of the size of albumin and of smaller molecular size with reasonable accuracy. We think it is better than any other relatively simple technique for studying the ratio between these two categories of serum proteins in urine. Because most proteins with a molecular size less than that of albumin can be measured as a group, the method is superior to a method in which a single protein can be measured.

This work was supported in part by NIH Grants AM 17196 and AM 17330. We thank Drs. K. V. Rao and J. P. Fidler for their help in obtaining urines for these studies.

References