
CLIN. CHEM. 30/3, 443–446 (1984)

Direct Analysis for Urinary Protein with Biuret Reagent, with Use of Urine Ultrafiltrate Blanking: Comparison with a Manual Biuret Method Involving Trichloroacetic Acid Precipitation
John H. Eckfeldt,1,2 Marcia J. Kershaw,1 and Irven I. Dahl1

We describe a method for measuring urinary protein with a centrifugal analyzer. Biuret reagent is used, and blanking with an ultrafiltrate of urine eliminates interferences from the nonprotein, biuret-positive chromogens in urine. We compare results by this new method with those by a manual method in which trichloroacetic acid precipitation and biuret reagent are used. The new method shows good precision and excellent correlation ($r = 0.997$) with the manual method. The ease and convenience of this assay should make this a useful method for the routine clinical laboratory.

Additional Keyphrase: centrifugal analyzer

Not all hyperproteinuria is clinically significant, but persistently high concentrations of protein in the urine may indicate renal or urinary-tract disease. Thus, detection and quantification of urinary proteins are important laboratory procedures (1), for which many methods have been devised. However, data from interlaboratory surveys show poor comparability of results within and among various quantitative methods (2–4), as well as frequent instances of poor intralaboratory precision. The methods most often used by clinical laboratories to quantify urinary protein are acid precipitation turbidimetry, protein dye-binding colorimetry, and biuret reagent with prior acid precipitation of protein (2–8). Alkaline benzathionium chloride precipitation turbidimetry (9) is also likely to be widely used as a consequence of its recent introduction for use in the Du Pont ace discrete analyzer. The major problems with all direct precipitation turbidimetric methods are the difficulty in obtaining reproducible and stable suspensions of the protein precipitate and the inaccuracies caused by interfering substances and variability in the composition of proteins appearing in urine (5, 6, 9–11). The Ponceau S and Coomassie Brilliant Blue dye-binding methods are also subject to inaccuracies caused by differences in urinary protein composition, although their precision is somewhat better than that of the acid precipitation turbidimetric methods (5, 6).

Biuret-based methods, being generally accepted as more nearly accurate, have been proposed as the basis for standardizing quantification of urinary protein (12). However, various nonprotein, biuret-positive interfering substances

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Received September 19, 1983; accepted November 28, 1983.
are a problem. Two general approaches have been used for their removal. Sephadex gel-filtration, to isolate the macromolecular components of urine, has been used in a Selected Method (13). Alternatively, the protein components of urine are precipitated with trichloroacetic or phosphotungstic acid, after which the acid-soluble interferences are removed in the decanted supernate (7, 8).

We have approached the problem of biuret-positive, low molecular-mass components in urine somewhat differently. Rather than trying to remove them before biuret colorimetry, we assay the urine with the interfering small molecules present and, in parallel, assay an ultrafiltrate of urine to quantify the contribution of these small molecules to the apparent urinary protein. We describe here this new approach and detail how results compare with those by a manual biuret method involving precipitation with trichloroacetic acid.

Materials and Methods

Materials

Reagents. Human albumin was from the American Red Cross Blood Services, Washington, DC 20006; human gamma-globulin from Cutter Biological, Emeryville, CA 94608; and albumin from Sigma Chemical Co., St. Louis, MO 63178. Pipacrilin (Pipracil) was from Lederle Pipracil, Inc., Carolina, PR 00630; nafcillin (Unipen) from Wyeth Laboratories, Inc., Philadelphia, PA 19101; and mezlocillin (Mezlin) from Miles Laboratories, Inc., West Haven, CT 06516. All other reagents were AR grade or better.

Samples. Samples were from 24-h urine specimens submitted for measurement of urinary protein. Samples were initially analyzed by the manual method (8), and then stored at 4 °C for no longer than 48 h before analysis by the present method.

Controls. Collect urine from normal subjects and store it refrigerated. Before analysis, filter through Whatman no. 42 filter paper to remove particulate material, add 1 g of sodium azide per liter, add an appropriate amount of bovine albumin for abnormal controls, and store 3-mL aliquots frozen at −20 °C. Such aliquots are stable for at least six months.

Procedures

Ultrafiltration procedure. Add 1.5 mL of centrifuged (1000 × g, 10 min) urine to a Centrifree® Micropartition system MPS-1 ultrafilter (prod. no. 4010; Amicon Corp., Danvers, MA 01923) equipped with an anisotropic, hydrophilic YMC ultrafiltration membrane (prod. no. 40420). This apparatus and membrane were originally designed for separation of low-molecular-mass compounds such as drugs from protein in serum. Centrifuge the sample in the ultrafiltration apparatus for 10 min at 1500 × g in a fixed-angle rotor to produce about 0.5 mL of ultrafiltrate.

Some lots of the YMT membrane contain a water-soluble, biuret-positive substance that elutes into the urine ultrafiltrate, causing spuriously high results for urinary interferences in the ultrafiltrate and, consequently, low calculated results for urinary protein. To resolve this problem, we soak all new membranes in de-ionized water for 15 min and blot before use. Membranes can be stored in 0.15 mol/L sodium chloride solution containing 2 g of sodium azide per liter and reused, but care must be taken not to damage or puncture them.

Biuret analysis procedure. Analyze the original urine and the above ultrafiltrate by a serum biuret method, increasing the sample volume by about 10- to 20-fold to compensate for the lower protein concentration in urine. We used a Multistat III Microcentrifugal Analyzer (Instrumentation Laboratory, Lexington, MA 02193), with the following reagents and procedure:

Biuret reagent: Instrumentation Laboratory’s Biuret Reagent Kit (prod. no. 35173), which contains, per liter, 333 mmol of sodium hydroxide, 66 mmol of potassium sodium tartrate, 50 mmol of potassium iodide, and 20 mmol of cupric sulfate.

Working urinary protein standard, 4 g/L: Dilute the 80 g/L bovine serum albumin standard provided in the reagent kit 20-fold with 0.15 mol/L sodium chloride solution containing 1 g of sodium azide per liter. This standard is stable for at least six months at 4 °C.

Load the Multistat III cuvette with samples and controls, using the following settings:

Sample syringe:
- Sample volume: 80 μL (80%) 80 μL (80%)
- Sample plus wash volume: 80 μL (80%)
Reagent syringe:
- Reagent volume: 125 μL (50%)
- Reagent plus wash volume: 125 μL (50%)
Reagent/diluent switch: reagent
Second-reagent switch: off

Place biuret reagent in the reagent boat. In the sample ring, place 200 μL of water in cup 1, 200 μL of urinary protein working standard in cup 2, and controls or patients’ urines and urinary ultrafiltrates in the remaining cups.

Place the loaded rotor into the analyzer. Parameters for the bichromatic endpoint analysis are: substrate II tape, program 42 (Total Protein); first filter, no. 7 (620 nm); second filter, no. 6 (550 nm); delay to first (620 nm) absorbance reading, 600 s; delay from first (620 nm) to second (550 nm) absorbance reading, 20 s; number of data points, 2; start mode 2 (no incubation to temperature before mixing).

Calculate the urinary protein concentration by subtracting the “protein” result for the urinary interferents in the ultrafiltrate (usually between 0.2 and 2.0 g/L, depending on the total 24-h urine volume) from the “protein” results for the unfiltered urine.

Results and Discussion

As shown in Table 1, the ultrafiltrate-blanked method for urinary protein has precision similar to that of the widely used quantitative urinary protein methods. Upon addition of 4 g of bovine albumin, human albumin, or human gamma-globulin per liter to 16 randomly selected, hospitalized patients’ urines, mean analytical recoveries were 99% (CV 6.1%), 98% (CV 7.0%), and 96% (CV 7.9%), respectively. The response of the method is linear with concentration to 10 g/L. Urines with apparent protein exceeding 10 g/L are very rarely encountered, but can be diluted with 0.15 mol/L saline before analysis. Depending on the instrumentation selected for protein analysis, precision in the low protein range may be improved somewhat by increasing the volume of urine and urinary ultrafiltrate relative to reagent volume. However, the range of method linearity is thereby truncated. Using this method primarily to follow patients with mild to moderate proteinuria, we chose assay conditions that would obviate frequent urine dilution, yet be reasonably precise in this concentration range for urinary protein.

As shown in Figure 1 and Table 1, the accuracy of the proposed method, as assessed by comparison with the trichloroacetic acid precipitation biuret method, is better than that for any of the dye-binding, acid precipitation turbidimetric, or alkaline benzathionium chloride precipitation.
Table 1. Representative Between-Batch Precision of Various Quantitative Urinary Protein Methods

<table>
<thead>
<tr>
<th>Protein, g/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
<th>n Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proposed ultrafiltrate-blanked biuret method:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal urine control</td>
<td>0.28</td>
<td>0.051</td>
<td>17.7</td>
<td>19 —*</td>
</tr>
<tr>
<td>Medium-protein urine control</td>
<td>0.89</td>
<td>0.054</td>
<td>6.1</td>
<td>24 —*</td>
</tr>
<tr>
<td>High-protein urine control</td>
<td>3.71</td>
<td>0.098</td>
<td>2.7</td>
<td>24 —*</td>
</tr>
<tr>
<td>Other widely used methods:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichloroacetic acid turbidimetry</td>
<td>0.62</td>
<td>0.046</td>
<td>7.4</td>
<td>10 6</td>
</tr>
<tr>
<td>Sulfosalicylic acid turbidimetry</td>
<td>0.62</td>
<td>0.070</td>
<td>11.3</td>
<td>10 6</td>
</tr>
<tr>
<td>Benzenethionium chloride turbidimetry</td>
<td>0.81</td>
<td>0.035</td>
<td>4.4</td>
<td>20 5*</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue</td>
<td>0.79</td>
<td>0.036</td>
<td>4.6</td>
<td>20 5*</td>
</tr>
<tr>
<td>colorimetry</td>
<td>0.62</td>
<td>0.087</td>
<td>14.0</td>
<td>10 6</td>
</tr>
<tr>
<td>Ponceau S colorimetry</td>
<td>0.72</td>
<td>0.035</td>
<td>4.9</td>
<td>20 5*</td>
</tr>
<tr>
<td>Trichloroacetic acid biuret</td>
<td>0.80</td>
<td>0.044</td>
<td>5.5</td>
<td>20 5</td>
</tr>
<tr>
<td>colorimetry</td>
<td>2.03</td>
<td>0.079</td>
<td>3.9</td>
<td>25 —*</td>
</tr>
</tbody>
</table>

*Data from our own laboratory.
*Correlation with trichloroacetic acid precipitation biuret method: \( r = 0.96 \) (5).
*Correlation with the trichloroacetic acid precipitation–biuret method: \( r = 0.98 \) (5).

![Graph](image)

Fig. 1. Comparison of the direct biuret, ultrafiltrate-blanked urinary protein method with the manual trichloroacetic acid precipitation, biuret urinary protein method (6)

Linear regression analysis of the data (\( n = 26 \)) gives a slope of 0.990 and an intercept of 0.05 g/L (\( r = 0.997 \)). The inset expands the data for urine with protein content < 1 g/L. For these data (\( n = 13 \)), the slope is 0.989, the intercept 0.11 g/L (\( r = 0.957 \)). The lines indicate perfect correlation, for comparison

Table 2. Interference with Urinary Protein Analysis by Penicillin-Type Antibiotics

<table>
<thead>
<tr>
<th>Apparent protein, g/L</th>
<th>Adult dose,*</th>
<th>% renally excreted</th>
<th>At minimum dose</th>
<th>At maximum dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>1–12</td>
<td>0.036 (neg.)</td>
<td>0 (neg.)</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>2–12</td>
<td>0.036 (neg.)</td>
<td>0 (neg.)</td>
<td></td>
</tr>
<tr>
<td>Nafcillin</td>
<td>2–12</td>
<td>0.036 (neg.)</td>
<td>0 (neg.)</td>
<td></td>
</tr>
<tr>
<td>Mezlocillin</td>
<td>6–18</td>
<td>0.036 (neg.)</td>
<td>0 (neg.)</td>
<td></td>
</tr>
<tr>
<td>Piperacillin</td>
<td>12–24</td>
<td>0.036 (neg.)</td>
<td>0 (neg.)</td>
<td></td>
</tr>
</tbody>
</table>

*Normal parenteral dose and approximate percent that is excreted unchanged in the urine (15, 16).

Another advantage of the ultrafiltrate-blanked method over direct acid precipitation turbidimetric and acid precipitation biuret methods is the absence of positive interference from certain renally excreted drugs; e.g., most penicillin-type antibiotics, which are excreted in large quantities. As shown in Table 2, some of these can significantly interfere with both qualitative and quantitative acid precipitation biuret-based methods, owing to their insolubility in acid solution and their formation of colored complexes in alkaline copper solutions. Although drug interference can sometimes be recognized by abnormal colors of the protein precipitate itself or after addition of biuret reagent, antibiotics have caused misdiagnosis of proteinuria in patients, which may be more common than is generally recognized (17, 18). With the proposed method, addition of similar concentrations of nafcillin, piperacillin, or mezlocillin to normal urine or urine supplemented with 5 g of pooled serum protein per liter merely increased the apparent "protein" in the ultrafiltrate blank and unfiltered urine equally, leaving the amount of measured urine protein unchanged.

In conclusion, the proposed ultrafiltrate-blanked biuret method for urinary protein is accurate, precise, and reasonably free of interferences; its ease and convenience should make it a very useful method for use in the routine clinical laboratory.

References
Quantification of Nonenzymically Glycated Albumin and Total Serum Protein by Affinity Chromatography

Randall W. Yatscoff, Gerald J. M. Tevaarwerk, and J. Courtney MacDonald

We have evaluated an affinity-chromatographic procedure for determination of glycated albumin (GA) and glycated total serum protein (GSP). Recovery of these analytes was inversely related to free glucose concentration, thus necessitating removal of free glucose. For this we used molecular-exclusion chromatography on G-25 Sephadex, or dialysis, the latter procedure resulting in significantly (p < 0.05) lower concentrations of GSP and GA. Total protein concentration and percent glycation are also inversely related, and so protein concentrations must be standardized before the assay. Within- and between-run CVs for both GSP and GA were <6.5 and 18%, respectively, the determination of GA being generally more precise of the two. Labile glycated fractions, lipemia, icterus, hemolysis, and type of anticoagulant did not affect the results, but assay temperature did. Diabetic subjects showed substantially higher concentrations of GA and GSP than did normal subjects. Because of the life span of these analytes in circulation, their measurement may provide a short-term index of glycemic control.

Additional Keyphrases: affinity chromatography • dialysis • diabetes • glycated hemoglobin • short-term index to control of glycemia • reference interval

In nonenzymic "glycation," the terminal amino group of glucose reacts with free amino moieties of protein, forming an aldimine–labile Schiff's base that subsequently undergoes an Amadori rearrangement to form a stable ketoamine adduct (1, 2). Of the many proteins that are susceptible to nonenzymic glycation, glycated hemoglobin has been the most widely studied (2, 3), primarily because of its usefulness as an index of long-term (two to three months) control of glycemia (2–4). A short-term (two to three weeks) indicator of glycemic control is also needed, for monitoring the effects of changes in diet or with insulin therapy (2). For this the determination of nonenzymically glycated albumin and glycated total protein in serum has been suggested (2, 5–17): their biological half-lives in the circulation are 17 and 30 days, respectively, compared with 120 days for hemoglobin (2).

Glycated albumin and total serum protein have been measured with ion-exchange and colorimetric procedures (2, 5–17). These methods are relatively imprecise, subject to interference from free glucose and enzymically glycated protein, and too time-consuming for routine use in a clinical chemistry laboratory.

Recently, an affinity-chromatographic method in which glycated proteins are separated from nonglycated proteins on the basis of interactions of glycoyl moieties with boronic acid has been adapted for measuring glycated hemoglobin (18, 19). Here we report our evaluation of a commercially available affinity method as an alternative means of quantifying glycated albumin and glycated total serum protein.

Materials and Methods

Specimens

At random times during the day blood was collected into tubes containing either EDTA or no anticoagulant, from laboratory personnel with no history of diabetes and from diabetic subjects who were being treated by diet, oral medication, or insulin. The samples were centrifuged at 1000 × g for 5 min to separate cells from serum or plasma. Hemolysates for determination of glycated hemoglobin were prepared as described below. All samples were stored at −80 °C until analysis.

Procedures

Quantification of glycated albumin and glycated total serum protein. Nonenzymically glycated albumin and total serum protein were quantified with a kit from Isolab Inc., Akron, OH, as follows.

1. Protein standardization and glucose elimination. Standardize the concentrations of total protein and albumin to 50 and 30 g/L, respectively, by adding appropriate volumes of isotonic saline. Then remove free glucose from the sample by gel filtration through a pre-packed Sephadex G-25 column (included in the kit) as follows. Add 200 μL of serum or plasma to the column, followed by 100 μL of sample wash buffer (per liter, 0.1 mol of MgCl₂, 0.1 mol of glycine, Triton X-100, and 0.1 g of sodium azide). Once the column drains, add 500 μL of wash buffer, and again allow to drain, discarding both eluates. Add 400 μL of wash buffer to the column, allowing the eluate to drip directly onto the top disc of the affinity column. Do not re-use these columns.

To eliminate free glucose by dialysis, use tubing having a

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Received August 15, 1983; accepted November 28, 1983.