Improved Biuret Procedure for Routine Determination of Urinary Total Proteins in Clinical Proteinuria

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This communication describes and evaluates an improved routine methodology for quantitating clinical proteinuria. Based on investigations of Piscator and of Savory et al., a modified Tsuchiya’s reagent (ethanolic HCl–phosphotungstic acid) is used to precipitate proteins at 56 °C, followed by biuret spectrophotometry at 540 nm. The accuracy of the proposed procedure was assessed by comparisons with results obtained by using an ultrafiltration membrane that retains solutes with an average molecular weight in excess of 10 000 for separating of urinary proteins before they are measured with the biuret reaction. Precision of the method (coefficient of variation) is typically 2–3%.

Additional Keyphrases: Tsuchiya’s reagent (ethanolic HCl–phosphotungstic acid) • spectrophotometry • ultrafiltration

Contemporary reports generally indicate that the total quantity of proteins in the urine of normal healthy adults averages less than 100 to 150 mg per liter (1). When the excretion is greater than about 250 mg per day, it is usually concluded that the subject has “clinical” proteinuria of potential pathological significance. More than 0.3 g per day indicates renal disease, unless associated with transient conditions such as proteinuria resulting from vigorous exercise. Accurate appraisal of clinical proteinuria is important not only for evaluation of certain renal diseases, but also for their early detection.

The status of determination of proteins in urine leaves much to be desired. It is estimated that a third of the clinical laboratories in the U.S. do not offer a quantitative procedure for urinary total proteins, and, of those that do, a fifth are using obsolete or inappropriate methods, and intra- and interlaboratory variation is high for those, that are using “reasonably good” methods.

The deficiencies of such analytical procedures, reviewed elsewhere (2), are such that it is enough to relate here that widely divergent results have been recorded repeatedly in comparative studies of various existing procedures (1, 2–7). Thus, a more reliable routine method for assessing clinical proteinuria clearly is needed.

In the present discussions “clinical proteinuria” should be interpreted medically as the presence of excess plasma proteins in the urine. It should be noted, however, that the proposed Tsuchiya’s reagent will precipitate the normal “Tamm–Horsfall” glycoprotein (8), which is the most abundant of the proteins derived from the urinary tract itself, as well as urinary proteins that originate from the plasma.

After testing several reported procedures from the literature, I concluded that Tsuchiya’s reagent (ethanolic HCl–phosphotungstic acid) for precipitating urinary proteins, followed by biuret spectrophotometry, was the most reliable. In 1923 Shevky and Stafford used this reagent to semiquantitatively estimate urine proteins (9), and it was later slightly modified (1, 10, 11). In the method described by Piscator (11), urine and reagent are allowed to react for 15 min at room temperature before removal of the precipitated proteins by centrifugation. Savory et al. (1) recommend precipitation for 15 min in an ice bath because they found that values for seven normal samples of urine averaged 6.1% (range, 2.8 to 10.2%) higher when precipitation was performed at 0 °C rather than 25 °C.

I decided to investigate precipitation of proteins from proteinuric samples at an elevated temperature, and I used 56 °C, for convenience, because most clinical laboratories have water baths or heating blocks adjusted to this temperature for various serological tests. I found that most urinary protein precipitates absorb less tan-colored pigments at 56 °C than 0 °C. Moreover, any color adsorbed during 15 min at 56 °C is more nearly removed by ethanol washing, resulting in both lower spectrophotometric blanks and less interference with the color produced by the biuret reac-
The typical magnitude of the statistically significant bias between precipitation at 0 and 56 °C was assessed with 10 urine samples. The data (Table 1) show that samples precipitated at 0 °C averaged 0.10 ± 0.11 (SD)g of protein/liter (range, -0.21 to +0.34; t = 2.86) higher than duplicates precipitated at 56 °C. That this significant difference is principally attributable to adsorbed urine pigments is further substantiated by the finding that corrected absorbance values for protein standards dissolved in physiological saline are unaffected by the temperature of precipitation. The heating step constitutes the most important improvement in the proposed methodology for proteinuric specimens.

Materials and Methods

Apparatus

I used a Bausch & Lomb “Spectronic 70” spectrophotometer with matched 1.27 cm (½-inch) round cuvettes. Comparative data were obtained by an ultrafiltration technique involving repetitive concentration and dilution of urine samples at room temperature by use of a nitrogen-pressurized stirred ultrafiltration cell, Model 12 (capacity, 10 ml), and “Diaflo” ultrafiltration membranes, type PM10, 25 mm diameter (both from Amicon Corp., 21 Hartwell Ave., Lexington, Mass. 02173). These membranes are composed of inert, nonionic polymers and retain solutes with an average molecular weight in excess of about 10 000. Pollak et al. (12) found protein recoveries to be excellent when serum or urine was concentrated through this membrane. Moreover, Diaflo ultrafiltration had little effect on the electrophoretic, immunoelectrophoretic, or quantitative immunodiffusion behavior of serum or urine proteins, confirming the results of Blatt et al. (13). Such urinary protein studies represent the continuance of numerous earlier investigations (14, for example) on the characterization of total nondialyzable solids of normal human urine, of which proteins form a part.

Reagents

Tsuchiya’s reagent, as modified by Lehmann (1). In a screwtop bottle containing 5.0 ml of concentrated hydrochloric acid, 6.0 ml of distilled water, and 77 ml of ethanol:water (95:5 by vol), dissolve 1.5 g of phosphotungstic acid.

Benedict’s qualitative solution, as cited by Piscator (11). In a beaker containing about 60 ml of distilled water, dissolve 17.3 g of sodium citrate dihydrate and 10.0 g of anhydrous sodium carbonate. In another beaker dissolve 1.75 g of cupric sulfate pentahydrate in about 10 ml of distilled water. Mix the two solutions and dilute the final reagent to 100 ml with distilled water. Stable indefinitely at room temperature.

Alkaline citrate “blank” solution. Prepare this reagent in the same manner as the Benedict qualitative solution, omitting the cupric sulfate. Stable indefinitely at room temperature.

Protein standard solutions. The biuret reaction given by a phosphotungstic acid precipitate of urinary proteins reportedly yields the same color intensity with an equivalent weight of albumin or globulins (1), as I confirmed with use of either bovine serum albumin, human serum albumin, or pooled human sera diluted appropriately with sodium chloride solution (8.5 g/liter) to achieve a known concentration of about 1.5 g/liter. “Abnormal Urine Chemistry Control” (Product No. 8920-80; Lederle Laboratories, Pearl River, N. Y. 10965), “2-Pak-Chemistry Urine Control, Supplemental” (listed No. 045-426; Hyland, Costa Mesa, Calif. 92626), or “Urine Control” (cat. No. U3001-2; Dade Division American Hospital Supply Corp., Miami, Fla. 33152) have served equally well as daily quality-control materials. These freeze-dried products are prepared from human urine and many constituents are assayed, including proteins in concentration of 0.9 to 1.5 g/liter. When 2.0 ml of standard or urine is used in the procedure to be described, Beer’s law is followed up to 2.0 g of protein per liter. Greater protein concentrations require more than 3.15 ml of biuret reagent for complete color production. The diluted protein standards are stable about one week at 4°C.

Procedures

Proposed routine biuret procedure for urinary total proteins.
1. Into one pair of test tubes or cuvets add 2.0 ml of centrifuged urine sample that contains not more than "24+" protein by qualitative testing (see "Calculations"). Into a second pair add 2.0 ml of protein standard solution.

2. Add dropwise, with mixing, 2.0 ml of Tsuchiya's reagent to all tubes, heat at 56 °C for 15 min, cool to room temperature with tap water, centrifuge for 10 min, carefully decant the clear supernatant liquids, and invert the tubes on filter paper to drain.

3. Add 1 ml of absolute ethanol to all tubes, disperse the protein pellets by means of a mechanical mixer, centrifuge the tubes rapidly for 5 min, decant the ethanol and again invert the tubes on filter paper to drain thoroughly.

4. Repeat step 3 once.

5. Add 3.0 ml of sodium hydroxide solution (30.0 g/liter) to all tubes, and mix to dissolve the washed protein precipitates.

6. Add 0.15 ml of alkaline citrate "blank" reagent to one of each pair of standard and urine duplicates, 0.15 ml of Benedict's solution to the other.

7. After mixing, leave the tubes at room temperature for 20 min for maximum development of the biuret color, which is stable for at least an hour.

8. Adjust the spectrophotometer to zero absorbance at 540 nm with a cuvet containing 3.0 ml sodium hydroxide solution (30.0 g/liter) plus 0.15 ml of alkaline citrate "blank" solution. Record the absorbances of the standard and sample pairs containing alkaline citrate "blank" reagent.

9. Adjust to zero absorbance with a cuvet containing 3.0 ml sodium hydroxide solution (30.0 g/liter) plus 0.15 ml of Benedict's solution. Record the absorbances of the remaining pairs, which contain Benedict's solution.

Calculations

Calculate "corrected" absorbances by subtracting the alkaline citrate "blank" absorbances (step 8) from the respective biuret absorbances (step 9).

Urinary total proteins (in g/liter) = (corr. A sample/corr. A std.) X concn. of std. soln. The sensitivity is about 20 to 30 mg/liter. If the protein concentration is greater than 2.0 g/liter, repeat the analysis, using 2.0 ml of urine previously diluted appropriately with water. A typical standardization graph, in which 2 ml of standard solutions of human serum albumin are used, is given in Figure 1.

**Comparative ultrafiltration-biuret procedure for urinary total proteins.**

1. Add 10.0 ml of centrifuged urine to the assembled ultrafiltration cell and ultrafilter the sample to a volume of about 0.1–0.2 ml.

2. Add 10 ml water to the cell and ultrafilter again as above.

3. Repeat step 2 twice.

4. Carefully add sodium chloride solution (8.5 g/liter) to the cell's 10-ml graduation mark, stir rapidly for about 15 s, promptly transfer the solution to a test tube, and centrifuge rapidly for about 5 min.

5. Pipet 2.0 ml of the centrifuged solution of urinary proteins into each of two cuvets and add 1.0 ml of sodium hydroxide solution (90.0 g/liter).

6. Complete the procedure and calculations as described above, starting with step 6.

**Results and Discussion**

The standard deviations for protein in three urine specimens, each determined 10 times each, but on separate days, by the proposed routine procedure, were ±40, 20, and 40 mg/liter; the coefficients of variation were ±2.0, 2.4, and 2.1%, respectively.

In another four-week study of day-to-day precision, 30 urine samples, each analyzed in duplicate on a different day, showed a CV of ±3.1% [1.50 ± 0.046 (SD) g/liter].

Duplicate recoveries of albumin solutions added to three proteinuric urine samples in final concentrations of 0.62, 1.51, and 2.34 g/liter averaged 102, 97, and 101%, respectively. Recovery of proteins from urine specimens is difficult to establish with certainty, because of the problems inherent in quantitating urine proteins accurately at low concentrations (12).

Accuracy was evaluated by measuring the protein concentrations of 11 urine samples by the proposed procedure and by the ultrafiltration methodology.
outlined above. Values by the ultrafiltration method averaged only 10 ± 49 (SD) mg/liter higher than the precipitation technique. The CV was ±3.4%, the standard error ±15 mg/liter. These correlation data establish that the routine procedure yields results that are not statistically significantly different from those obtained by the more laborious ultrafiltration. In this investigation no quantitative measurements of proteins in normal urine were undertaken. However, 2-ml portions of 10 urine samples giving “negative” results with sulfosalicylic acid were carried through the procedure. The “corrected” absorbances ranged from 0.000 to 0.010 (mean, 0.003). If desired, the method may be adapted to studies of normal urine by analyzing a larger volume of sample (20 ml), as proposed by Savory et al. (I).

Proposals for standardization of total protein assays in serum as detailed by Peters (15) state “total protein shall be defined as the total nondialyzable (molecular weight greater than 10,000) polypeptide substances.” Also, because results for serum by biuret techniques are known to correspond closely with values obtained by the Kjeldahl technique, specification of an established version of the biuret reaction, with use of standards prepared from either bovine or human serum albumin, is advised for intra-laboratory use (15, 16). Savory et al. (I) preferred a standard solution of pooled human sera for their studies on normal urine.

Although reports on serum to date (15, 16) do not include specific discussion of urinary total proteins, it would seem desirable to adopt the same analytical principles as far as possible. This communication describes and evaluates such a routine technique for assessing clinical proteinuria. I believe that the method provides a valid basis for comparison and investigation of other procedures for urinary “total proteins.” For example, a brief preliminary study indicates that the acid-acetone method (17) for precipitating urinary total proteins (including mucoproteins) generally may yield similar values. However, even if further data should support this tentative observation, the method described here is more convenient.

References


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