that precedes it). The authors expect the D-dimer test to be useful in the evaluation of other hemorrhage extravascular fluids as well.

References


Mildly increased urinary albumin excretion rates and concentrations, below the quantity normally detected by conventional urinary protein and albumin methods, have prognostic significance for the development of nephropathy in patients with diabetes mellitus. The authors evaluated the automated Behring Nephelometer using Behring reagents for the detection of low level urinary albumin. Within run coefficients of variation (CVs, N = 20) are 1.7%, 1.3%, and 2.4% at mean urinary albumin levels of 16, 70, and 217 mg/L, respectively. Between run CVs (N = 20) are 4.5%, 2.6%, and 4.4% at mean albumin levels of 19, 71, and 239 mg/L, respectively. The method is sensitive to

3 mg/L. Hemoglobin, immunoglobulins, bilirubin, urea, and radiographic contrast media beyond a few hours of injection show no significant interference at levels normally expected from clinical specimens. Analysis is unaffected by pH within the physiologic range. Most urine specimens are stable for at least eight days when refrigerated at 4 °C. Specimen centrifugation before analysis is essential to avoid a negative bias that occurs when analyzing uncentrifuged refrigerated samples. Preanalytical freezing produces results higher than those observed in fresh or refrigerated samples. The authors conclude that automated nephelometry using the Behring Nephelometer is a convenient, simple, and accurate technique for the determination of low level urinary albumin. (Key words: Nephelometry; Urinary protein; Proteinuria; Diabetes mellitus) Am J Clin Pathol 1990; 93:405-410
DIABETIC NEPHROPATHY is a serious and frequent complication of diabetes mellitus, particularly in patients with insulin-dependent (type I) disease. More than 40% of insulin-dependent diabetes mellitus patients will develop nephropathy during the course of their disease. In the United States, roughly 25% of the 91,000 dialysis patients are diabetic. Low levels of urinary albumin, below the concentrations detected by urinary dipstick (e.g., Albustix®) and conventional urinary protein methods, are prognostically significant for nephropathy progression in diabetes mellitus patients. \( \text{Furtherm} \), strict metabolic and hypertension control slows or reverses the progression toward overt diabetic nephropathy. \( \text{Therapeutic intervention, however, is most effective in the early, subclinical stages of diabetic renal impairment; once the patient has sufficient renal dysfunction to produce albuminuria detectable by conventional means, little can be done to reverse the process.} \) Low level albuminuria has been referred to in the literature by many terms, including microalbuminuria, \( \text{incipient} \) \text{diabetic nephropathy, elevated urinary albumin excretion, slight albuminuria, and albuminuria.} \) \text{Many techniques exist to detect subclinical albuminuria, including radial immunodiffusion, enzyme immunoassay, dye binding assays (nonspecific proteinuria), and immunonephelometric and immunoturbidimetric procedures.} \( \text{Most reported techniques are either imprecise or relatively time consuming. Nephelometry offers the advantage of acceptable precision, possibility for automation, and ease of analysis.} \) An immunonephelometric assay using the Behring Laser Nephelometer recently was reported, offering excellent precision and semiautomatic operation. \( \text{Urinary albumin determination using the new Behring Nephelometer has been briefly reported by Behring Diagnostics and collaborators in Europe.} \) \text{This study reviews our findings, compares them with those from Europe, and discusses modifications we have made to the standard protocols that facilitate analysis.} \)

Materials and Methods

Instrumentation

The Behring Nephelometer (Behring Diagnostics, Inc., Somerville, NJ) quantifies light scattering induced by antigen-antibody complexes precipitating in aqueous media. The instrument uses an 840-nm high-performance infrared light emitting diode light source and converts detected scattered light to a voltage reading. This represents a change from the previous Behring Laser Nephelometer, which used laser light at 632.8 nm. The new instrument also performs automatic dilutions as necessary to obtain optimum light scatter on the linear portion of the Heli-delberger/Kendall curve. Furthermore, reagents have been optimized to reduce lengthy assay incubation times, affording expedient result reporting.

Reagents and Assay Procedure

A 100 \( \mu \text{L} \) sample (standard, control, urine) is mixed, undiluted, with 40 \( \mu \text{L} \) of pure antihuman albumin antiserum and 160 \( \mu \text{L} \) of buffer in a reusable reaction cuvette. An initial reading is taken at 10 seconds and a final reading at 6 minutes. The accelerated fixed time method used in this analysis differs from other nephelometric determinations that use a rate method. The difference in light scatter between these readings is compared against a curve generated from known human albumin standards. Albumin concentrations were analyzed in 100 mg/L increments from 100 to 1,000 mg/L to identify the urinary albumin level that would produce antigen excess (postzone), and thus incorrectly yield falsely low results.

We obtained the albumin standard (N-Protein Standard, 41.50 g/L albumin), rabbit antihuman albumin and phosphate buffered saline (PBS, N-Diluent) from Behring Diagnostics, Inc. Two antisera lots were available to evaluate lot-to-lot antisera variation. Human immunoglobulin purified from Cohn Fractions II and III was obtained from Sigma Chemical Company (St. Louis, MO). Immunoglobulin purity was reconfirmed by electrophoresis using a Beckman agarose gel (Beckman Instruments, Brea, CA), stained with amido black (Beckman Instruments). Ames Bili-Labstix® (Miles Laboratories, Inc., Elkhart, IN) were used to screen urine for protein (albumin), bilirubin, and hemoglobin. Hemoglobin A was obtained from Helena Laboratories (Beaumont, TX), and the human albumin standard used to test assay linearity was obtained from Quantimetrix (Hawthorne, CA) (20 g/L). This standard was selected because our laboratory previously had referenced it against a now discontinued College of American Pathologists second standard.

The N-Protein standard was manually diluted 1:20 with PBS to give a preassay working standard concentration of 2.075 g/L. We used a six-point calibration curve for the assay; the instrument diluted the working standard 1:40 to 1:1280 to yield an assay range of 1.6 to 51.9 mg/L for undiluted samples. Although the manufacturer initially recommended a seven-point calibration curve, including a 1:2,560 dilution (0.8 mg/L standard), the quantity of light scattered at this concentration was sufficiently small, to, at times, reject that calibration point and the entire calibration curve. We consider a protein concentration of 1.6 mg/L sufficiently sensitive for detecting low level urinary albumin and thus elected to use the six-point calibration curve, eliminating the 1:2,560 dilution. The instrument manufacturer subsequently released software that automatically performed a 1:5 dilution before the
initial analysis, using a six-point curve. Since the software performed analyses within a measuring range of 9.5 to 270 mg/L, we elected to use an open channel to program the instrument to first analyze specimens undiluted, allowing analysis in the 1.6 to 9.5 mg/L range.

Linearity was evaluated with the Quantimetrix human albumin standard diluted 1:66 to 1:8,000, giving a concentration range of 2.5 to 303 mg/L. The 303 mg/L concentration was selected as a maximum concentration because higher values constitute overt proteinuria and can be detected by conventional urinary protein methods.

Three controls for within run and between run assays were selected. The low and midrange controls were prepared by combining urine from healthy donors with that from overt albuminuric patients. The third control was prepared by diluting a commercial urine control 1:4 with deionized water giving the desired concentration (Level II urine chemistry control, Fisher Laboratories, Orangeburg, NY). The pH effect on the assay was determined by adjusting a voluntarily donated urine sample with hydrochloric acid or ammonium hydroxide giving urinary pHs within the physiologic range. Hemoglobin interference was evaluated for pure hemoglobin and for erythrocytes. For interference by pure hemoglobin, the Helena hemoglobin standard was diluted to approximate concentrations of 5.0 g/L, 25 mg/L, 5 mg/L, 2 mg/L, and 1 mg/L, which gave dipstick results of 3+, 3+, 2+, 1+, and trace, respectively. To test for red cell interference, washed red cells were combined with urine samples to give concentrations of 1.0%, 0.5%, and 0.25% packed cells, by volume. Each sample was then lysed by repeated freezing and thawing. All red cell concentrations were reflected by 3+ dipstick results. Radiographic contrast media (iothalamate meglumine, 600 g/L [Conray] (Mallinckrodt, Inc., St. Louis, MO) was studied in urinary concentrations from 18 to 108 g/L. Iothalamate meglumine since these concentrations may be expected in urine based on the half-life of the drug that is not metabolized and is renally excreted. Three samples (10, 20, and 100 mg/L albumin) were prepared in urea concentrations up to 4 mol/L and stored at 4 °C. Aliquots of each sample were analyzed for eight days. A frozen aliquot was analyzed on the eighth day. Possible bilirubin interference was studied by evaluating albumin recovery from dilutions of an icteric urine specimen (2+ by Bili-Labstix®).

To test for effects of preanalytical specimen centrifugation and for specimen stability, samples were stored at 4 °C and analyzed for one week. Aliquots of the same samples were frozen, stored for one week at −20 °C, thawed and then assayed to determine the effect freezing has on specimens. Analysis was concurrently performed initially on centrifuged and uncentrifuged urine. Subsequently, once centrifugation appeared to yield more reliable results, all stability studies were performed on cen-

trifuged urine. Centrifugation was at 350 X g for 5 minutes.

All interference and linearity analyses were performed in duplicate to reduce the random variation inherent in the procedure.

Results

Linearity and Accuracy

Acceptable results were observed for urinary albumin levels ranging from 1.6 to 300 mg/L. Based on the initial diluted value 200 mg/L (1:100 dilution), from which subsequent samples were prepared, values obtained were within 4 mg/L of the expected value, up to 200 mg/L. Between 0 and 25 mg/L, the critical assay range, results obtained were repeatedly within 1 mg/L of expected values. At 300 mg/L, a 5–6% positive bias was observed (15–18 mg/L), although this difference is of less clinical significance at this higher level. In one of three linearity studies, an approximate 10% positive bias was present between the theoretical calculated value and the instrument analyzed value. This approximate 10% bias between the observed and expected values based on the Quantimetrix human albumin standard used most likely results from the large initial manual dilutions necessary to reduce the serum protein standard to concentrations representative of those found in urine.

Stability and Centrifugation

Sufficient interference was observed in the analysis of uncentrifuged samples following refrigeration that the instrument was, at times, unable to provide a sample result. Investigation revealed the initial reading at 10 seconds was outside the instrument’s acceptable limits. This interference increased over one week, although some samples demonstrated interference with one day’s storage. Centrifugation yielded acceptable interassay precision when analyzing refrigerated samples for durations up to eight days.

Initially, 2 of 20 samples with low urinary albumin showed significant increases in measured albumin concentration when stored at 4 °C and at −20 °C for eight days. The first sample increased from 1.9 mg/L initially to 5.0 mg/L (−20 °C storage) and 5.2 mg/L (4 °C storage) when measured at day 8. Similarly, the second sample rose from 6.2 mg/L to 13.9 mg/L (−20 °C) and 14.9 mg/L (4 °C) at eight days. Less variation was observed at 4 °C when the study was repeated using urine specimens obtained from the above two individuals and five additional volunteers. Four of these seven urines, when stored at −20 °C for 8 days, yielded increased urinary albumin concentrations, up to 65% higher when compared with initial assay concentrations. A third study, using a human
albumin standard at concentrations of 10, 20, and 100 mg/L, failed to demonstrate this effect. Recovery at 8 days in this third study was 94–98% when stored at 4 °C and 88–98% when stored at −20 °C.

We performed a subsequent study on 56 random patients to further evaluate stability when specimens are stored at 4 °C. While we did observe a positive trend over an eight-day period, the increases were within the error of the method.

Precision and Interlot Antisera Variation

The intraassay and interassay precisions, shown in Table 1, were equal to or better than those reported in the literature for immunonephelometric and other assays. The findings are comparable with those reported by the manufacturer and collaborators in Belgium. Ten patient urine samples, ranging in concentration from 5.7 to 85.9 mg/L, showed excellent correlation (R = .999) when assayed with two different lots of antisera.

Analysis Limits

The instrument consistently interpreted urinary albumin concentrations between 52 and 400 mg/L as being outside the 1.6 to 51.9 mg/L analysis range and performed the specimen dilution necessary for result calculation and interpretation within the standard curve’s linear range. Urinary albumin concentrations at or above 700 mg/L were consistently misinterpreted as being within the instrument’s analytical range. The 500 and 600 mg/L results were incorrectly yielded low results because of antigen excess.

Interferences

Results obtained from analysis of a 12.9 mg/L sample showed no interference across a pH range of 5.36 to 9.76. Similarly, interference was not observed with the pure hemoglobin standard and the human immunoglobulin within the concentration ranges evaluated. Lysed packed red cells showed a 16% positive interference only with analysis of the 1.0% packed cell sample. We were unable to document consistent interference from hematuria, up to 2+ by dipstick. Radiographic contrast media gave at least a 9% negative bias when present in concentrations at or exceeding 108 g/L and a 4% bias at 72 g/L.

Bilirubin failed to show any interference up to semiquantitative concentrations of 2+ determined by Bilistix®. Urea up to 3 M did not interfere with the assay; a 2 mg/L (20%) decrease in albumin concentration was observed when the 10 mg/L sample was assayed in 4 M urea.

Discussion

Our evaluation demonstrates urinary albumin excretion can be routinely quantified by the technique described. This new instrument offers the advantage of a considerably shorter incubation time when compared with methods previously reported. Furthermore, the current method allows for urinary specimen analysis without prior manual predilution, minimizing the inherent random error thus introduced. Preanalytical specimen centrifugation appears to be a requirement for analysis of undiluted specimens. We reanalyzed several uncentrifuged specimens that the instrument considered unsatisfactory due to a high initial reading, instructing the instrument to make a 1:5 dilution before analysis. Although this modification allowed the instrument to analyze these specimens, a negative bias was observed. We postulate this negative bias results from the settling or dissolution of suspended particles between the initial 10-second and the final 6-minute readings.

Urinary albumin concentrations in the overt albuminuria range may be misinterpreted by the instrument because of antigen excess. We believe this technique should be offered to quantify low level urinary albumin while existing techniques (e.g., urinary total protein and electrophoresis) be used to quantify frank proteinuria. We, therefore, prescreen all urines submitted for low level urinary albumin analysis with a dipstick sensitive to albumin. Urinary dipsticks (e.g., Labstix®, Albustix®) are sensitive to 50–80 mg/L and become 1+ above 300 mg/L. We consider any urine with an albumin dipstick value less than or equal to 1+ acceptable for analysis by our technique. Screening allows for detection of those specimens that may give falsely low readings due to antigen excess, although we perform an “antigen excess” check on all urines with 1+ protein by programming a mandatory 1:5 dilution for these samples. This protocol eliminates analysis from patients with overt clinical albuminuria.

Controversy remains regarding effects of specimen storage. We observed a trend toward increasing urinary protein concentrations when clinical specimens were studied; similar findings were not observed when a human albumin standard was used. Elving and colleagues found a decrease in measured urinary albumin concentration
when patient specimens were stored at -20 °C for two and six months. We agree with Elving and colleagues’ conclusions that urine specimens should be stored a 4 °C and analyzed as soon as possible. Although Elving and colleagues recommend analysis within two weeks of collection, our results suggest analysis should be performed within eight days. This fits well into a weekly batch processing schedule.

Although red blood cells in the urine may interfere with analysis, such interferences appear to be present only at red cell concentrations in excess of those usually found in clinical specimens. When red blood cells or hemoglobin is present, however, the possibility of a vascular source cannot be excluded. Thus, a positive hemoglobin by dipstick suggests blood (red cells and serum) may be present in the urine. Although even a small amount of serum may increase measured urinary albumin, we were not consistently able to document this occurrence. We recommend caution when interpreting specimens with hematuria and suggest the etiology for the hematuria be ascertained and the condition resolved, if possible, before quantitative urinary albumin analysis is performed.

References to urinary radiographic contrast media concentrations normally found in clinical specimens could not be located; however, we examined those concentrations that seemed likely on the basis of the drug’s half-life and its excretion route. Concentrations above 72 g/L did introduce a negative bias. Such concentrations, while possible in clinical practice, would be unexpected in urine collected more than 3 hours after injection of iothalamate meglumine. Therefore, we recommend urine for analysis not be obtained from patients on the day when a procedure using contrast media is performed; these patients should void before collection for urinary albumin begins.

Controversy exists in the literature regarding the optimum specimen for low level urinary albumin and the best method for reporting results. While some authors believe a spot urine to be adequate as an initial screen, most recommend a timed collection ranging from several hours to 24 hours. Some centers report only a urinary albumin concentration (i.e., mg/L albumin), whereas others prefer a urinary excretion rate (i.e., µg/min albumin).

One of the most important applications for testing patients for low level urinary albumin is to follow an individual patient over time to evaluate the progression of diabetic nephropathy. In this context, the patient serves as his own control. It is important, therefore, in the evaluation of any one patient, that the collection and reporting methods be the same over time. The authors have elected to report the urinary albumin concentration and the urinary albumin excretion rate for each specimen analyzed. The authors recommend a 24-hour timed specimen but will accept random or other timed collections. Diurnal variation exists for urinary albumin excretion and varies with activity. Early morning collections show the best reproducibility, so the authors recommend an early morning collection when a 24-hour specimen is uncollectable. Including the collection time in the report allows intra-individual standardization across time.

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References


Rapid Immunoperoxidase Demonstration of Rickettsia rickettsii in Fixed Cutaneous Specimens from Patients with Rocky Mountain Spotted Fever

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Immunofluorescence (IF) of skin biopsies for detection of Rickettsia rickettsii (RR) has proven useful as a rapid test for confirmation of Rocky Mountain spotted fever (RMSF). However, IF lacks sensitivity, requires special equipment and training, and is difficult to interpret. The authors have developed an indirect avidin-biotin immunoperoxidase (IP) technique to detect RR in fixed and frozen tissue sections. The technique was evaluated on fixed cutaneous specimens from patients dying of RMSF and compared to specimens from control patients dying of an acute febrile illness with skin manifestations and vasculitis. IP correctly identified RR in 9 of 12 cases with probable identification in 2 additional cases. Of 11 controls, 10 were negative and one was uninterpretable. RR were easily visualized within cytoplasm and nuclei of endothelial cells in association with perivascular lymphocytic infiltrates and less frequently with vasculitis or noninflamed vasculature. IP is rapid, amplifies small quantities of antigen, gives excellent histologic detail as compared with IF, and is easily adapted for use in hospitals with immunoperoxidase capabilities. (Key words: Rocky Mountain Spotted Fever; Rickettsia rickettsii; Immunoperoxidase diagnosis; Vasculitis; Endothelium) Am J Clin Pathol 1990;93:410–414

ROCKY MOUNTAIN SPOTTED FEVER (RMSF) continues to occur in many geographic regions of the United States, and despite the availability of effective antibiotics, mortality has remained at between 3 and 6%. Even when classic symptoms of fever and rash are present, the remaining clinical presentation can be quite variable and nonspecific.

Attempts to design tests for early diagnosis of RMSF have revealed the usefulness of immunofluorescent stain...