Nephelometric Methods for the Determination of Urinary Albumin, Transferrin and Alpha-2 Macroglobulin*

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ABSTRACT

Measurement of the urinary excretion or clearance of certain plasma proteins as an index to glomerular permeability has been the subject of numerous investigations. Most of these studies, however, have employed semi-quantitative analytical techniques. The precise determination of several proteins associated with glomerular proteinuria should provide useful clinical information regarding the integrity of the glomerular filtration apparatus. This report describes immunochemical methods for the determination of three urinary proteins which are increased in glomerular proteinuria: albumin, transferrin and alpha-2 macroglobulin.

Introduction

Estimation of protein in urine has long been used in the evaluation of renal disease. Although many systemic disorders may result in proteinuria, most work on the quantitation of urinary proteins has been directed toward characterization of protein excretion and/or clearance patterns as they relate to glomerular permeability or tubular reabsorption. Increased glomerular permeability gives rise to enhanced urinary excretion of proteins the size of albumin or larger.

Numerous techniques have been introduced for the measurement of albumin and other proteins in urine. In 1914, Folin and Denis reported a method for urinary albumin utilizing sulphosalicylic acid. Since that time, various immunochemical procedures have been studied. The turbidimetric method of Schultz and Schwick was adapted to urinary albumin measurements in 1961 and pre-concentration of the sample was required. A concentrated urine is also needed for the detection of albumin, transferrin, and alpha-2 macroglobulin by immunoelectrophoresis or radial immunodiffusion. Radioimmunoassay procedures also possess the sensitivity for quantitation of urinary proteins, but this approach has not been extensively studied.

Recent studies in our laboratory have led to the development of several manual procedures for the measurement of immunochromical reactions in aqueous solutions. This paper describes adaptations of a nephelometric technique to the measurement of specific proteins in urine.
Principle

Urinary albumin, transferrin and alpha-2 macroglobulin were determined by measurement of light scattering from the immunochemical reaction between each specific protein and the appropriate goat antiserum. Under the condition of antibody excess, the light-scattering response from the reaction was proportional to the concentration of the protein in the urine sample. Commercial reference sera, diluted to concentrations expected in urine, were used for standardization.

Reagents

**Phosphate buffered saline (PBS).** A phosphate buffer (ph 7.4, 10 mmol per liter) was prepared by dissolving 11.80 g of Na$_2$HPO$_4$ and 2.33 g of NaH$_2$PO$_4$ in one liter of distilled deionized water. Particulate matter was removed from the solution by passage through a filter with a 0.22 µm (average pore size) grid. The buffer was then diluted 10-fold with filtered normal saline (9 g per l) and used as a diluent for all reagents, samples, and standards.

**Goat antihuman albumin, transferrin, and alpha-2 macroglobulin.** Goat antisera for each protein were obtained for use in the assay procedures. The albumin antiserum (batch #A402-2) and transferrin antiserum (batches #A424-1 and #A424-2) were diluted 25-fold with PBS prior to use. In order to maximize the sensitivity of the procedure for alpha-2 macroglobulin, this antiserum (batch #A417-1) was only diluted 10-fold. Particulate matter was removed from the diluted antisera by filtration. Storage at 5° proved adequate to maintain antiserum potency.

Standard Solutions

**Specific Protein Standards**

Standardization was accomplished by the use of commercial reference sera, diluted with PBS to concentrations expected in urine. Each serum had been assayed by the commercial laboratory against accepted primary standards. The albumin reference serum (batch #D711-1) contained 566 mg of albumin per dl and was diluted to give standards of 37.5, 21.2, 9.4, and 1.4 mg per dl. Standards of 7.2, 5.1, 3.6, 1.2, and 0.45 mg per dl were prepared from the transferrin reference (batch #D755-1, 360 mg per dl). Dilutions of the alpha-2 macroglobulin serum (batch #D741-1, 380 mg per dl) provided standards of 7.6, 5.4, 3.8, 1.9, and 0.4 mg per dl. Undiluted reference sera and diluted working standards were stored at 5° with no apparent change after several weeks.

**Apparatus**

The light-scattering measurements were made with a fluorescence spectrophotometer equipped with a square quartz flow cell, which had a volume of about 0.5 ml. The wavelength for the incident light was set at 468 nm, which corresponds to the maximum intensity of the xenon source lamp. A cut-off filter was placed between the excitation monochromator and the sample cell in order to eliminate second-order excitation. The secondary monochromator was set at 470 nm so as to exclude non-specific fluorescence. Other instrumental conditions, such as slit width and sensitivity setting, were adjusted so as to obtain optimum results with each antigen-antibody system.

An automatic pipette was employed to aspirate the urine samples, mix them with one ml of the appropriate antiserum, and deliver the reactions mixtures into test tubes for incubation. The pipet was equipped with a five ml diluent pump and a 50 µl sample pump for the albumin determina-

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* Millipore Corp., Bedford, MA 07130.
† Catalogue number 176F-QS, Hellma Cells, Inc., Jamaica, NY 11424.
‡ Micro Medic Systems, Philadelphia, PA 19105.
TABLE I
Dilutions for Urine Protein Measurements

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reaction Mixture</th>
<th>Sample Blank Mixture</th>
<th>1:1 Dilution Mixture</th>
<th>Antiserum “Blank” Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reaction Mixture</td>
<td>Sample Blank Mixture</td>
<td>1:1 Dilution Mixture</td>
<td>Antiserum “Blank” Mixture</td>
</tr>
<tr>
<td></td>
<td>Urine Sample (ml)</td>
<td>Diluted Antiserum (ml)</td>
<td>Urine Sample (ml)</td>
<td>PBS (ml)</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.02</td>
<td>1.00</td>
<td>0.02</td>
<td>1.00</td>
</tr>
<tr>
<td>Transferrin</td>
<td>0.05</td>
<td>1.00</td>
<td>0.05</td>
<td>1.00</td>
</tr>
<tr>
<td>Alpha-2 Macroglobulin</td>
<td>0.05</td>
<td>1.00</td>
<td>0.05</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Procedures

All urine samples were initially tested with Albustix* in order to provide an estimate of the concentration of total protein content. Those samples exhibiting severe proteinuria were prediluted to help insure an antibody excess in the reaction. Samples containing large amounts of particulate matter were filtered to avoid interference with the light-scattering measurements.

In table I are shown the reaction conditions established for measurement of albumin, transferrin and alpha-2 macroglobulin in urine. In the albumin procedure, 20 µl of urine were drawn up into the automatic pipette and then delivered with 1.0 ml of diluted antiserum into a 10 × 75 mm disposable test tube.† After the tubes were sealed with Parafilm and mixed 10 times by inversion, they were allowed to stand at room temperature for at least 20 minutes. The reaction was shown to reach equilibrium after 10 minutes with antigen and antibody in approximately equivalent proportions (figure 1). Light scattering from the reactions and 1:1 dilution mixtures was measured in the fluorescence spectrometer after the delay period. None of the samples assayed for albumin demonstrated measurable blank light scatter so that blank corrections were not necessary before determining albumin concentrations from the

Figure 1. Light scattering as a function of time for the albumin—anti albumin system.

Figure 2. Average standard curve for urine albumin measurement.

* The Ames Company, Elkhart, IN 46514.

† Kimble Products, Toledo, OH 43601.
standard curve. In figure 2 is depicted the average standard curve from a series of measurements made during one week and shows the fluctuation of each standard during that time period.

A 1:1 dilution antigen excess screen was especially important when making urine protein measurements since concentrations can vary so greatly in renal disease. The dip-stick screen was quite useful in detecting samples with high total protein levels so that appropriate dilutions could be made. It may also be noted that the standard curve reached well above the normal range of urine albumin allowing the quantitation of a wide range of concentration without tedious predilution steps.

The procedures for urine transferrin and alpha-2 macroglobulin measurement were similar to that described for albumin. Owing to the very low concentrations of these proteins in urine, some modifications were necessary to attain maximum sensitivity. The sample size in both cases was increased to 50 µl, and the alpha-2 macroglobulin antiserum was diluted only 10-fold. The larger sample size resulted in small but significant blanks. Therefore, blank corrections were made prior to determinations from the standard curves. In addition, a delay period of two hours was required to achieve equilibrium in these procedures. The modifications were sufficient to substantially lower the limits of detection for transferrin and alpha-2 macroglobulin but not enough to measure either protein in normal urine. These procedures should be useful, however, in the evaluation of patients with various stages of proteinuria.

Results and Discussion

Several factors must be considered in the immunochemical quantitation of proteins in urine which do not pertain to measurements in either serum or cerebrospinal fluid. Primary among these are the effects of urea and pH on the immunochemical reaction. Urea concentration and pH can be expected to fluctuate over a wide range in normal and pathological urine samples. In recent studies on a model system, urea was shown to inhibit the antigen-antibody reaction to a greater extent than hydrogen ion concentration, but both could be expected to cause inaccurate results. Dilution of the urine sample in buffer was determined to be the most simple and efficient means of eliminating the effects of these two factors. In the routine assay procedures, the urine samples were diluted 50-fold for albumin measurements and 20-fold for the analysis of either transferrin or alpha-2 macroglobulin. These dilutions reduce the concentration of urea well below those expected to inhibit the immunochemical reaction, and the pH is stabilized by the buffer. No apparent inhibition of the reactions by urea or pH was observed in the present study.

Detection of fluorescence from drugs or drug metabolites found in urine was eliminated by setting excitation and emission monochromators at about the same wavelength. Under these conditions, only scattered light was detected.

The precipitin curve for the measurement of urinary albumin (figure 3) was
constructed using dilutions of the reference serum ranging from 1.4 to 45.4 mg per dl. The limit of detection of this method (1 mg albumin per dl) included values of albumin found in normal urine, and, as stated above, antibody excess extended to concentrations almost thirty times the upper limit of normal. The wide range of concentrations included in the antibody excess region of the precipitin curve made this procedure ideal for screening apparently normal subjects as well as for making precise, quantitative albumin determinations on patients with kidney disease.

Transferrin levels have been reported in normal urine at concentrations ranging from 0.011 to 0.042 mg per dl, and alpha-2 macroglobulin has been detected as a "trace." As shown by the precipitin curves for transferrin (figure 4) and alpha-2 macroglobulin (figure 5), neither of these proteins could be detected in normal urine by the present procedures. The limits of detection of the two procedures (0.45 mg transferrin per dl, and 0.4 mg alpha-2 macroglobulin per dl) were determined by the dilution of the urine sample. Less than 20-fold dilution of the samples led to inhibition of the reactions by urea and could have caused inaccurate results in some cases. A compromise was achieved in an attempt to reduce the effects of urea and at the same time develop a method with adequate sensitivity for the proteins of interest in pathological samples. Concentration of urine samples by readily available commercial apparatus should make possible the detection of these proteins at their normal levels. Transferrin, however, was easily detected in the unconcentrated urine of patients with mild proteinuria. Alpha-2 macroglobulin was present in measurable levels in patients with heavy proteinuria.

The precision of the urine protein methods was assessed by performing replicate

<table>
<thead>
<tr>
<th>Protein</th>
<th>n</th>
<th>SD (mg/dl)</th>
<th>Mean (mg/dl)</th>
<th>RSD* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>20</td>
<td>0.20</td>
<td>15.9</td>
<td>1.30</td>
</tr>
<tr>
<td>Transferrin</td>
<td>15</td>
<td>0.04</td>
<td>1.79</td>
<td>2.36</td>
</tr>
<tr>
<td>Alpha-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macroglobulin</td>
<td>20</td>
<td>0.14</td>
<td>3.37</td>
<td>4.23</td>
</tr>
</tbody>
</table>

* Relative standard deviation.

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The precision of the urine protein methods was assessed by performing replicate
analysis on pooled urine samples. These precision data are presented in table II and indicate excellent reproducibility for all three procedures. These precision data may be attributed to the use of an automatic system for all sample and reagent pipetting. This automatic system also reduced the time required for pipetting and thereby increased the efficiency of the procedures, making them more suitable for use in the clinical laboratory.

Sources of Error

Antiserum Specificity

Monospecificity of the antiserum used must be established by immunological techniques prior to use as an analytical reagent. Precipitin curves should be characterized for each batch of antiserum since specificity and potency varies between batches.

Reaction Inhibition

Urea and hydrogen ion concentration have been shown to inhibit the immunochromical reaction. Inhibition may be minimized by dilution of the urine sample in a phosphate buffer of pH 7.4.

Non-specific Fluorescence

Fluorescence from drugs or drug metabolites found in the urine may be eliminated by adjusting fluorometer excitation and emission monochromators to the same wavelength. Under these conditions, only scattered light is detected.

Normal Ranges

The normal range for albumin, transferrin and alpha-2 macroglobulin are listed in table III.

Résumé of Clinical Interpretations

The glomerulus is known to exhibit selective permeability to plasma proteins in various disease states. Proteinuria is classified as selective when relatively small amounts of high molecular weight proteins are excreted. Nonselective proteinuria results in the excretion of larger amounts of high molecular weight proteins. Measurements of several urine proteins with different molecular weights can therefore provide indices of selectivity which are related to the type and severity of the renal lesion.5,6,11,12,19

References

13. Petersson, P. A., Evrin, P. E., and Berggard, I.: Differentiation of glomerular, tub-


