

Screening for Microalbuminuria by Use of a Rapid, Low-Cost Colorimetric Assay

G. Phillipou, S. K. James, C. J. Seiborn, and P. J. Phillips

We evaluated the pyrogallol red–molybdate(IV) method for quantification of urinary protein as a screening procedure for microalbuminuria by determining the assay’s sensitivity and specificity at different concentrations of urinary albumin as measured by a comparison laser-nephelometric immunoassay. The pyrogallol red–molybdate(IV) method has sensitivity and specificity similar to that for other semiquantitative assays, but it is less expensive and sample throughput can be high if microtiter plate techniques are used.

Additional Keyphrases: pyrogallol red–molybdate(IV) • diabetic nephropathy

Small increases in albumin in urine of patients with diabetes are associated with subsequent development of clinical nephropathy and retinopathy (1–3). "Microalbuminuria" is generally defined as urinary albumin corresponding to \( >20 \mu g/min \) or approximately \( 30 \text{ mg/L} \) (4). The most appropriate upper limit, however, has not been unequivocally established. Detection of microalbuminuria in patients with diabetes is important, because deterioration of renal function may be reversible if it is detected at the early stages (4).

Low concentrations of albumin in urine can be accurately quantified by immunochemical methods. However, these methods are not suitable for screening, because they have a limited calibration range, require trained laboratory staff, and have a high cost per sample. Accordingly, many centers use qualitative or semiquantitative techniques to initially screen urine specimens for subsequent quantitative immunnoassay (5–10).

Recently Watanabe et al. (11) reported a colorimetric method for the quantification of urinary protein in the range \( 10–1000 \text{ mg/L} \). The procedure is simple to perform and the reagents are inexpensive. We have adapted their method to microtiter plates and correlated results with a nephelometric immunoassay for urinary albumin, similar to that reported by Marre et al. (12). This adaptation has produced a rapid, reliable, and low-cost method to select clinic patients for further assessment of microalbuminuria.

Materials and Methods

Materials: Albumin (Cohn Fraction V) and pyrogallol sulfonphthalein were from Sigma Chemical Co., St. Louis, MO. The protein assay reagent was prepared exactly as described by Watanabe et al. (11). Rabbit antiserum to human albumin (SRI titer: 2.25 mg/L) was from Dakopatts, Gloetrup, Denmark.

Instrumentation: Nephelometric measurements were performed with a Behring Laser-Nephelometer (Behringwerke AG, Marburg, F.R.G.). Plates were read with a Titertek plate reader (Flow Laboratories, Nth Ryde, Sydney, Australia), set at 620 nm.

Urine specimens: These were untimed specimens collected from patients attending the diabetic outpatients clinic. Although untimed daytime specimens may not be optimal for investigation of microalbuminuria (however, see Watts et al. (13) and Nathan et al. (14)), they provide a suitable compromise for initial selection when large numbers of clinic patients are to be repeatedly screened. Samples are mixed by hand, then centrifuged at 5830 \( \times \) g for 2 min before being stored at \(-20^\circ \text{C} \) for no more than three days before assay.

Pyrogallol red–molybdate assay (PR method): Standards consist of albumin in concentrations of 0, 25, 50, and 100 mg/L in distilled water containing sodium azide, 1 g/L. This range of standards was selected to increase the precision of estimation at the critical lower concentrations of protein where interference is most likely.

Controls are selected patients’ urines. Into the appropriate well of the microtiter plate, 25 \( \mu L \) of urine, standard, or quality-control sample is placed. Urines and quality-control samples are assayed in duplicate; standards are assayed in quadruplicate. The pyrogallol red reagent (200 \( \mu L \)) is then quickly added and the plate allowed to stand at room temperature for 30 min before the absorbance at 620 nm is measured.

The standard response is analyzed by least-squares regression and the derived function parameters are used to calculate the respective concentrations of urinary protein. The intra-assay CV for a urine quality-control sample was estimated at 5.8% \( (n=37); \text{mean} = 49.8 \text{ mg/L}; \text{SD} = 2.9 \). The interassay CV was 4.1% \( (n=15); \text{mean} = 48.4 \text{ mg/L}; \text{SD} = 2.0 \). The CV for the total assay is therefore 7.0%.

Endocrine and Diabetes Laboratory, The Queen Elizabeth Hospital, Woodville, South Australia 5011.

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Nephelometric albumin assay: The antiserum is diluted 25-fold with a 40 g/L solution of polyethylene glycol 6000 in pH 7.4 phosphate buffer. Quality controls include a selected urine sample, stored at −20 °C, and the Pharmacia Albumin Controls (LH; Pharmacia Aust. Pty. Ltd., Nth Ryde, Sydney, Australia). Urines for which the initial value exceeds 130 mg of protein per liter are diluted 10-fold with distilled water, those with >550 mg/L are diluted 20-fold. All samples are analyzed in duplicate.

The assay is performed in 1-mL disposable plastic cuvettes, which are initially tested in the nephelometer to determine their respective blank value. To each cuvette is added 100 µL of urine, standard, or quality-control sample, followed by 200 µL of antiserum. After 60 min the cuvettes are re-read in the laser-nephelometer. The response is the difference between the 60-min and blank values.

The urinary albumin concentration is calculated by evaluating the response (y) with respect to a polynomial function of the form \( y = a + (b \cdot x) + (c \cdot x^2) + (d \cdot x^3) + (e \cdot x^4) \), where \( x \) is albumin (mg/L) and \( a, \ldots, e \) are derived constants. The latter function was established from results obtained by repeated analysis (\( n = 15 \)) of albumin standards in concentrations of 0, 2, 5, 10, 15, 30, 50, and 100 mg/L in phosphate buffer, pH 7.4, to which Triton X-100 surfactant (1 mL/L) had been added to prevent adsorption of albumin onto the plastic (15). The 15 mg/L albumin standard is routinely included in every assay as a calibration check. At 0.6 mg/L albumin, the assay signal/noise ratio is 10.2. Comparison with an immunoturbidimetric assay (16) gave the following regression equation: turbidimetric = 2.0 + 0.957 \cdot nephel (\( r = 0.939; n = 26 \)).

The assay quality-control sample had a mean of 18 mg/L (\( n = 13 \)) with intra- and interassay CVs of 2.4% and 3.5%, respectively. Assay means for the Pharmacia controls were 3.9 mg/L (total CV = 9.3%; \( n = 12 \)) and 24.6 mg/L (total CV = 8.6%; \( n = 12 \)), respectively. The values stated by Pharmacia ranges for their RIA assay are 3.2 mg/L (SD = 0.27; interassay CV = 8.4%) and 24.3 mg/L (SD = 1.5; interassay CV = 6.2%).

Results and Discussion

The pyrogallol red method, originally reported by Fujita et al. (17), measures the shift in the absorption spectrum of the pyrogallol red–molybdate(IV) complex when protein is bound. We originally attempted to apply this method to quantify urinary protein accurately in the range 5 to 30 mg/L, but were unsuccessful, owing to a consistent negative sample-matrix bias. Watanabe et al. (11) resolved this problem by adding sufficient oxalate (1 mmol/L) in the reagent to ensure saturation of all endogenous urinary chelators. This type of modification, however, increases the detection limit of the assay to approximately 20 mg of protein per liter. The reference interval for healthy adults, as measured by the pyrogallol red method, has been reported as 28–141 mg/24 h (11). Guder and Heiland (18) cite 40–100 mg/24 h for dye-binding methods in general.

The characteristic blue complex that forms in the pyrogallol red method gradually precipitates on long standing, particularly at higher protein concentrations, and can be first seen in the plate well at 120 to 150 mg/L. The blue color also provides a method by which the rare high "false-positives" (>150 mg/L) caused by interferents such as fluorescent dyes may be readily detected.

The overall correlation between results by the present and nephelometric assays for 179 diabetic patients was 0.93.

For measured protein values of <200 mg/L—a more important range for the purposes of this study—the correlation coefficient was 0.74 (\( n = 141 \)). The latter comparison is shown in Figure 1. A representation of all the values by the colorimetric pyrogallol red method with respect to critical cutoff values determined by the nephelometric assay is summarized as a histogram plot in Figure 2. With the absolute discriminant concentration set at <30 mg of albumin per liter as measured nephelometrically, Figure 3 shows the respective sensitivity and specificity curves for
Table 1. Sensitivity and Specificity of the Present Method at Different Albumin Concentrations

<table>
<thead>
<tr>
<th>Albumin, mg/L</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
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<tbody>
<tr>
<td>NIA* 80</td>
<td>87.1</td>
<td>89.1</td>
</tr>
<tr>
<td>NIA* 60</td>
<td>98.6</td>
<td>75.5</td>
</tr>
<tr>
<td>NIA* 50</td>
<td>100.0</td>
<td>58.2</td>
</tr>
<tr>
<td>NIA* 300</td>
<td>94.1</td>
<td>91.4</td>
</tr>
<tr>
<td>NIA* 30</td>
<td>98.0</td>
<td>82.8</td>
</tr>
<tr>
<td>NIA* 60</td>
<td>100.0</td>
<td>65.8</td>
</tr>
<tr>
<td>NIA* 50</td>
<td>90.0</td>
<td>98.7</td>
</tr>
<tr>
<td>NIA* 400</td>
<td>96.7</td>
<td>95.3</td>
</tr>
<tr>
<td>NIA* 100</td>
<td>100.0</td>
<td>93.3</td>
</tr>
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*Urinary albumin as measured by the nephelometric assay. * Urinary protein as measured by the present method.

The present method satisfies many requirements for initial screening of patient samples for microalbuminuria. It is simple, inexpensive, sensitive, specific, rapid, capable of high throughput, and provides an accurate guide to the dilution required for subsequent immunoassay.

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References