Development of an affordable dye-stained microalbuminuria screening test

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Abstract

Background. A simple spot test was developed, which allows quantification of microalbuminuria. Evaluation was carried out according to the ISO 15189 guidelines.

Methods. Urine was spotted on cellulose acetate strips and stained using different sensitive protein binding dyes (nigrosin, Coomassie Blue R-250, amido black). The colour intensity of the stained spots was quantified using a Kodak Image 450 station.

Results. Analytical sensitivity of the Coomassie Blue based method (18 mg/L) was better than that for nigrosin (50 mg/L) or amido black (100 mg/L) based methods. Within-run coefficient of variation (CV) and between-run CV of the Coomassie blue assay were, respectively, 8.4% and 9.7% (50 mg/L), and 3% and 4.5% (400 mg/L). For nigrosin, these data were, respectively, 8.4 and 9.4 (50 mg/L), and 3% and 4.5% (400 mg/L). Coomassie Blue showed a preferential binding selectivity towards albumin. The method was found to be linear between 20 and 600 mg/L. A good correlation ($r^2 = 0.89$) was obtained between Coomassie Blue based and immunonephelometric measurements. Immuno-unreactive albumin (prepared by protease treatment) could be detected by the spot test, which offers an advantage of the method versus immunochemical tests. Ammonium sulphate precipitation could further increase the specificity of the assay by eliminating effects of free light chains.

Conclusion. The described method is very simple and extremely cheap, which makes it potentially suited for screening programmes, particularly in third world countries.

Keywords: Coomassie brilliant blue R-250; immuno-unreactive albumin; microalbumin; nigrosin; screening

Introduction

Urinary albumin levels between 20 mg/L and 200 mg/L are considered as an early marker for glomerular damage [1]. Microalbuminuria is an excellent early marker of...
Furthermore, the possibilities for further improving the analytical performance of the various dyes-based recipes will help in the selection process to find the optimal method to fulfil clinical needs for a microalbuminuria screening test. Therefore, regular screening for microalbuminuria should be a standard of follow-up in diabetics, hypertensives and prenatal care in clinical practice.

At present, quantitative measurement of microalbuminuria is only possible by immunochromatographic or chromatographic techniques [3]. It has been reported that conventional immunoassays underestimate the urinary albumin concentration because albumin in urine may exist in two forms, one immuno-reactive and the other immunochemically unreactive. Under physiological conditions, filtered albumin is fully degraded by tubular cells. In the case of tubular damage, albumin will be degraded to smaller fragments. This immunochemically unreactive albumin is not detectable by radio immunoassay and immunoturbidimetry [4].

In developing countries, particularly in Sub-Saharan Africa, the prevalence of diabetes is rapidly rising. By 2006, 10 million people were suffering from diabetes and this figure is expected to increase to almost 20 million by 2025. Although the rest of the world is also experiencing a global epidemic of diabetes, the biggest growth rate and impact of this disease will be in the developing countries [5]. Because of lack of financial and technical facilities, the local population has not sufficient access to microalbuminuria screening or to a routine monitoring and health care. Therefore, the development of an affordable microalbuminuria screening test could mean a useful contribution in the management of diabetes and hypertension in these low-income countries. Kutter et al. [6] have already demonstrated the analytical performance and practicability of (semiquantitative) screening tests based on sensitive dyes.

In this study, we want to explore the possibilities of developing sensitive dye-binding microalbumin methods in combination with quantitative reading. In particular, Coomassie Blue R-250, nigrosin and amid black staining methods will be investigated and evaluated according to the ISO 15189 guidelines [7]. Results will be compared with those obtained through immunonephelometry, which can be regarded as a standard technique [8]. Comparing the analytical performance of the various dyes-based recipes will help in the selection process to find the optimal method to fulfill the clinical needs for a microalbuminuria screening test. Furthermore, the possibilities for further improving the analytical selectivity using salt precipitation will be explored.

Materials and methods

Staining

Two microlitres of random [fresh (<2 h) or stored at 4°C] urine specimens or standard solution were spotted on GA 19372 cellulose acetate sheets (Fluka Biochemika, Buchs, Switzerland) measuring 25 × 150 mm. Upto 14 urine samples could be spotted on a single sheet. Calibration was carried out using human serum albumin (Dade Behring, Marburg, Germany) saline solutions ranging from 20 to 200 mg/L.

Nigrosin solution was prepared by dissolving 1 g of nigrosin, 15 g of sulphasalicylic acid and 15 g of trichloracetic acid in 500 mL water. Destaining was carried out in water by gentle agitation.

Solutions of Coomassie Brilliant blue R-250 were prepared by dissolving 1 g of Coomassie R-250 (Sigma, St Louis, MO, USA) in 225 mL of methanol, 50 mL acetic acid and 225 mL water. Destaining was carried out in 5% acetic acid for 2 min, followed by further destaining in a mixture containing 450 mL of denaturated ethanol, 100 mL of glacial acetic acid, and 450 mL of water.

Amido black solution was prepared by dissolving 1.5 g of amido black in a mixture containing 25 mL water, 125 mL methanol and 25 mL glacial acetic acid. Destaining occurred in 5% acetic acid.

Quantification of the spots

Following destaining and drying the cellulose acetate strips to the air, the density of the stained spots was quantified on a Kodak Image 450 image station (Eastman Kodak, Rochester, USA) according to the manufacturer’s guidelines. No optical filter was used. The mean intensity of the stained spot was used as a parameter for determining the concentration.

Comparative analysis

Microalbuminuria was measured immunonephelometrically on randomly selected patient samples (n = 51) with use of commercially available Dade Behring antibodies on a Behring Nephelometer II analyser (Dade Behring, Marburg, Germany) standardized against the widely accepted WHO/College of American Pathologists Certified Reference Material 470.

Total urinary protein was assayed by a pyrogallol red method [9] with Standard Reference Material 917a as a standard and commercially available reagents (Instruchemie BV, Delfzijl, The Netherlands) on a Modular P system (Roche, Mannheim, Germany).

Specificity of the stain

Electrophoresis (300 V, 20 min) of diluted (1:200 in 0.1 mol/ L phosphate buffered saline, pH 7.4) control serum (Sebia, Evry, France) was carried out on the same cellulose acetate strips followed by dye staining and destaining according to the procedure described for the microalbumin determination. The capillary electrophoresis of the control serum followed by UV densitometry at 200 nm (Capillaries, Sebia) was considered as a reference [10]. The median relative ratios (experiment was run in 10-fold) between the percentages of the stained electrophoretic fractions (albumin, alpha 1, alpha 2, beta and gamma globulins) and the ones obtained by capillary electrophoresis were calculated to determine the specificity of the Coomassie R-250 and nigrosin staining procedures.

Ammonium sulphate precipitation followed by filtration was carried out for salting out free light chains and other
globulins according to Blondheim [11]; 3 g of ammonium sulphate (Merck, Darmstadt) were added to 5 mL of urine and filtered on a Whatman filter paper. The obtained filtrate was then further used for analysis.

**Sensitivity—Linearity**

The lower limit of detection [12] was calculated as the mean value $+ 3 \sigma$ for a blank sample. Linearity was evaluated in the range between 15 and 2000 mg/L. Linearity was assessed according to NCCLS [13].

**Reproducibility—Analytical performance**

Three human urine pools with a microalbuminuria of, respectively, 50 mg/L, 100 mg/L and 200 mg/L obtained from routine samples with known microalbuminuria were used to assess the precision of the assay. The within-day variance was determined as the average of the variances obtained at each day ($n = 10$). The between-day variance was calculated from the variance of the means obtained each day ($n = 10$), which was then adjusted for the within-day variance component.

**The effect of urinary pH**

The effect of urinary pH was evaluated by comparing the spot intensity of the standard solution (final concentration: 100 mg/L) that was dissolved in various buffer solutions: 0.1 mol/L acetate buffer (pH 4.7); 0.1 mol/L phosphate buffer (pH 7.3); 0.1 mol/L veronal buffer (pH 8.8). These experiments were carried out in 5-fold.

**Analysis of immuno-unreactive albumin**

Immuno-unreactive albumin was prepared by protease treatment. Fifty microlitre of a protease (source: Streptomyces griseus, Sigma, St Louis, USA) solution [10 mg/mL diluted in 0.1 mol/L phosphate buffered saline (0.048 mol/L Na$_2$HPO$_4$, 0.02 mol/L KH$_2$PO$_4$, 0.145 mol/L NaCl, 0.015 mol/L Na$_3$PO$_4$, pH 7.2)] was added to urine samples containing albumin (0.35 g/L). Following enzymatic digestion (30 min, 1 h, 2 h at room temperature), the urine specimen was re-analysed for microalbumin using both immunochromatography and dye-binding methods.

**Statistics**

$P$-values $< 0.05$ were considered significant. The agreement between dye-binding methods and immunochemiluminescence was evaluated by Spearman rank analysis.

**Results**

**Performance**

Lower limit of detection was 18 mg/L for the Coomassie Blue method and 50 mg/L for the Nigrosin method. For the amido black method, values of 100 mg/L were found. Because of the disappointing analytical sensitivity, no further analytical validation of the amido black staining was carried out. All stains showed a comparable linearity in the range up to 500 mg/L. Typical standard curves for Coomassie Blue R-250 and nigrosin are depicted in Figures 1 and 2. Figure 3 illustrates the images obtained by both methods in a dilution series (starting concentration: 500 mg/L). Table 1 summarizes the results of the within-run and between-run coefficients of variation (CV) for both dye-binding methods.

**Specificity towards various proteins**

The specificity of Coomassie Blue R-250 and Nigrosin staining towards the various protein fractions was compared. Table 2 summarizes these results. Coomassie Brilliant Blue R-250 has a marked affinity towards albumin, whereas nigrosin staining is much less specific ($P < 0.0001$), and shows also a high affinity towards gamma globulins. Variation of urinary pH in the range 4.7–8.8 did
**Discussion**

Amido black staining was not sensitive enough to be taken into account for developing a sensitive microalbumin assay, which is in agreement with earlier literature [14]. The described Coomassie Brilliant Blue R-250-based microalbumin method was found to be more sensitive than the nigrosin- and the amido black-based methods. The measuring range and the sensitivity of this method meet the analytical criteria for microalbuminuria testing [15]. Although the reproducibility of the test is worse than the reproducibility of immunoturbidimetry or immunonephelometry [8], the reproducibility is clinically satisfactory in view of the high biological variability of the analyte that shows a within-subject variation of 36% [16]. The marked affinity of Coomassie Blue R-250 for albumin resulted in a high specificity towards albumin that is in agreement with earlier literature [14]. The marked affinity of Coomassie Blue R-250 for albumin resulted in a high specificity towards albumin that is in agreement with earlier literature [14]. The marked affinity of Coomassie Blue R-250 for albumin resulted in a high specificity towards albumin that is in agreement with earlier literature [14].

Nigrosin staining was found to be much less specific for albumin and shows an additional marked affinity towards gamma globulins making this method more prone to analytical interferences originating from non-glomerular types of proteinuria (tubular proteinuria and free light chain proteinuria) [1].

**Immuo-unreactive albumin**

Following protease treatment, microalbumin results were little affected in the spot tests. Following a 1 h protease digestion, respectively, 92 ± 6 and 82 ± 15%, of the initial concentration was recovered for the Coomassie Brilliant Blue R-250 and the nigrosin-based method (Table 3). In contrast, the immunochemical albumin assay showed a rapid decrease of albumin immunoreactivity ($P < 0.001$ after 30, 60 and 120 min).

**Table 3.** The effect of protease treatment on the various albumin assays

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>Coomassie</th>
<th>Nigrosin</th>
<th>Immunonephelometry</th>
</tr>
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<tbody>
<tr>
<td>30</td>
<td>103 ± 3</td>
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<tr>
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<td>120</td>
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</tbody>
</table>

$^{*}P < 0.05; \ ^{**}P < 0.01; \ ^{***}P < 0.001$. 

**Method comparison**

We also found good agreement between the immunonephelometric microalbumin method ($\times$) and Coomassie Blue R-250 based determination: $y$ (microalbumin) mg/L = 0.670 × (nephelometric determination) + 45 (mg/L); $r^2 = 0.945; \ P < 0.001$. Agreement between the immunonephelometric microalbumin method ($\times$) and the nigrosin-based determination was worse: $y$ (microalbumin; mg/L) = 0.640 × (microalbumin, nephelometric determination; mg/L) + 53; $r^2 = 0.800; \ P < 0.001$.

**Table 1.** Within ($n = 10$) and between ($10$-days)-run coefficient of variation (CV) for Coomassie and Nigrosin-based methods

<table>
<thead>
<tr>
<th>Albumin (mg/L)</th>
<th>Coomassie</th>
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<tr>
<td></td>
<td>Within-run CV (%)</td>
<td>Between-run CV (%)</td>
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</tr>
<tr>
<td>50</td>
<td>8.4</td>
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</tr>
<tr>
<td>100</td>
<td>5.2</td>
<td>8.7</td>
<td>5.3</td>
<td>9.0</td>
</tr>
<tr>
<td>200</td>
<td>3.0</td>
<td>4.5</td>
<td>3.4</td>
<td>6.4</td>
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**Table 2.** Specificity of Nigrosin and Coomassie Brilliant Blue R-250 towards various plasma protein fractions

<table>
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<th>Protein fraction</th>
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<tr>
<td>Nigrosin</td>
<td>0.66 (0.60–0.72)</td>
<td>0.41 (0.30–0.51)</td>
</tr>
<tr>
<td>Coomassie</td>
<td>1.42 (1.31–1.55)**</td>
<td>0.08 (0.03–0.13)</td>
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**Relative staining intensity (average protein = 1)**

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<td>Alpha 1 globulin</td>
<td>0.28 (0.22–0.38)</td>
<td>0.16 (0.09–0.21)</td>
</tr>
<tr>
<td>Alpha 2 globulin</td>
<td>0.55 (0.40–0.70)</td>
<td>0.50 (0.41–0.61)</td>
</tr>
<tr>
<td>Gamma globulin</td>
<td>3.57 (3.10–4.10)</td>
<td>0.55 (0.48–0.62)</td>
</tr>
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**Relative staining intensity (average protein = 1)**

**Fig. 3.** Comparison between nigrosin (above) and Coomassie R-250 (below) based techniques. The figure depicts the spots obtained by both methods in a 1:2 dilution series (starting concentration: 500 mg/L; upper-left corner).

**Immunofluorescence microscopy**

In a series of urine samples containing free light chains ($n = 6$), a mean concentration of the M-protein 2.4 ± 0.9 g/L, ammonium sulphate precipitation followed by filtration was able to eliminate the interference of these proteins.

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</table>
The correlation of the Coomassie Brilliant Blue R-250-based method with the standard immuno-nephelometrical method in clinical samples was acceptable. As can be expected from the specificity data, the correlation between nigrosin-based method and the immunochemical albumin determination is weaker. Urinary pH did not affect test results, which corresponds with earlier literature data [19]. In contrast to the immunochemical microalbumin assay [4], the dye-binding-based tests also pick up the immuno-unreactive albumin. The development of colour in Coomassie dye-based assays has been associated with the presence of basic amino acids (primarily arginine, lysine and histidine) in the protein. Van der Waals forces and hydrophobic interactions also participate in the physical adsorption of the dye by protein. The type of binding makes the dye-binding assays more robust in certain diabetics, presenting with a high concentration of immuno-unreactive albumin [4].

In the case of doubtful results, due to the presence of excessive amounts of free light chains, addition of ammonium sulphate to the urine sample followed by filtration allowed us to eliminate the analytical interference of free light chains. The ammonium sulphate procedure, therefore, can be used for confirmation purposes.

Since the described dye-binding method is technically simple and does not require the use of antibodies, the cost of the assay is very low (based on actual prices on the African market, the estimated cost of a dye-binding test is very suitable for screening purposes, particularly in poorer and warmer countries.

Acknowledgement. This work was financially supported by the VLIR-UOS project ZEIN2007SEL18-127.

Conflict of interest statement. None declared.

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cessed)
Diagnostic accuracy of a reagent strip for assessing urinary albumin excretion in the general population

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¹Laboratorio di Analisi Chimico Cliniche ed Ematologiche, Ospedale Civile Maggiore and ²Dipartimento di Scienze Biomediche e Chirurgiche, Divisione di Nefrologia, Università degli Studi di Verona and Ospedale Civile Maggiore, Azienda Ospedaliera di Verona, Italy

Abstract

Background. Albuminuria is a sensitive marker of renal derangement and has been included in a number of studies investigating chronic kidney diseases (CKDs). This study is aimed to evaluate the diagnostic performances of a strip for measuring the albumin/creatinine ratio (ACR) in the general population and to compare it with those found in a diabetic population.

Methods. Urine samples were obtained from 201 consecutive subjects enrolled in an epidemiological study and from 259 type 2 diabetic patients. Urine was tested for albumin and creatinine using the strip (Clinitek Microalbumin) and laboratory methods. A hundred samples were stored under various conditions to assess analyte stability.

Results. In the general population, the strip test reached a 90% sensitivity and 91% specificity, considering the laboratory method as the ‘gold standard’, sparing >80% of subjects the laboratory tests at the expense of a 1% false negative rate and an 8% false positive rate. Regarding sensitivity and specificity, the ACR test performs very similarly in the general population and in the diabetics. The stability study showed that storage at −20°C induced a significant decrease in the albumin concentration with both methods, such that 5% of the samples were re-classified in the lower ACR class. Storage at −80°C for up to 12 months did not affect the measurement with both methods.

Conclusion. Clinitek Microalbumin strips can be used for screening purposes in the general population since they correctly classify a significant percentage of subjects, particularly those with a normal albuminuria. Storage at −80°C does not affect strip results. Screening with the strip and confirming positive results with a wet chemistry method does not affect strip results. Screening with the strip and confirming positive results with a wet chemistry method are an efficient strategy for detecting albuminuria in the general population.

Keywords: albumin/creatinine ratio; CKD; diabetic nephropathy; dipstick urinalysis; screening

Introduction

While the burden of chronic kidney disease (CKD) and end-stage renal disease is increasing dramatically in both developed and developing countries, most subjects in the earlier stages of kidney disease go undiagnosed and undertreated [1–3]. Finding solutions for renal disorders demands strategies not only to prevent the adverse outcome of kidney

doi: 10.1093/ndt/gfn639
Accepted in revised form: 24.11.08