Performance indicators and validity of different analytical methods for measuring urine protein and microalbumin in patients with diabetes mellitus

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ABSTRACT

Objectives: 1. To derive a reference range for random urine protein-creatinine index (PCI) and albumin: creatinine index (ACI) in apparently healthy subjects, using different analytical methods.
2. To compare and assess the validity of sulfosalicylic acid (SSA) and pyrogallol red (PGR) methods for measuring urine protein as alternatives to immunoturbidimetric (IT) method for measuring microalbumin, in Iraqi diabetics with or without microalbuminuria (MA) or renal proteinuria.

Subjects and Methods: Random and fasting blood specimens were collected from 400 diabetic (256 females, 144 males) aged 8-87 years, including 48 type 1 and 352 type 2 diabetic. They were attending Al-Waffa Diabetic Clinic in Mosul during 6 months from 1st August 2002 to 31st January 2003. A control group of 415 apparently healthy volunteers (108 females, 37 males) aged 15-72 years were used for comparison. Urine protein was measured using SSA and PGR (for all diabetics and controls) and urine albumin using IT (for 112 diabetics and 75 controls). The statistical methods used included unpaired student t-test and linear regression analysis. The validity indicators: sensitivity, specificity, negative and positive predictive values and accuracy rate were calculated.

Results: The frequency distribution of PCI and ACI showed log-normal distribution and following log transformation, the reference range for PCI was 20-235 mg/g using SSA and 18-205 mg/g using PGR, and for ACI was 4.55 mg/g using IT. The overall prevalence of proteinuria in the diabetics was 30% using SSA method and 35% using PGR method and MA was 27%.

The SSA and PGR methods for measuring proteinuria were compared with IT method for measuring MA. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy rates were 93%, 96%, 90%, 98% and 95.5% respectively for PGR, and 95%, 95%, 95%, 93% and 91% respectively for SSA. A highly significant correlation (P < 0.001) was observed between ACI and PCI values (r = 0.85 using PGR, r = 0.88 using SSA) in all control and diabetic subjects. Prediction of ACI from PCI value can be made by multiplying the PCI value by 0.375 using PGR and 0.39 using SSA.

Conclusion: Proteinuria and MA are common among Iraqi diabetics. Simple and cheap methods, particularly the PGR method, have acceptable performance to be routinely implemented in diabetic care. It is recommended to measure random urine PCI in all diabetics during their regular visits to the diabetic clinic.

Keywords: Microalbuminuria, sulfosalicylic acid, pyrogallol red.
Diabetes mellitus can lead to long-term complications of microangiopathic and macroangiopathic origin. One of these complications is nephropathy which is considered to be a major cause of morbidity and mortality. It is no secret that according to a WHO report, its prevalence after 15 years of diabetes is 17.7-56.6% in men and 11.9-71.1% in women.

Measurement of urine albumin excretion is used for the early diagnosis of diabetic nephropathy and for monitoring the effectiveness of treatment. Several approaches for urine sampling have been recommended including 24 hr, overnight, short-term and random urine collections. Measurement of albumin or protein concentrations alone or in relation to creatinine concentration (expressed as albumin: creatinine index, ACI; or protein: creatinine index, PCI) has been proposed.

Random urine sampling is simpler and easier to 24 hour collection and the ACI or PCI are strongly correlated with timed excretion, making these measurements suitable and convenient alternatives to timed urine collection particularly, for follow up and screening purposes.

Many methods for measuring protein or albumin in urine have been reported. However, none of the methods is completely satisfactory, and all assays suffer from standardization problem. The currently available methods include dip stick testing as well as qualitative and quantitative techniques.

**MATERIALS AND METHODS**

The 545 subjects who participated in this study were divided into 2 groups:

1. Control group (Group 1): This group consisted of 145 apparently healthy volunteers (108 females, 37 males), age range 15-72 years with mean ± SD of 32.3 ± 14.2 years, and body weight 38-107 Kg, 68.1 ± 14.8 Kg. One hundred subjects were the residents of Al-Hmedeat village, 25 Km North West of Mosul center. The subjects were chosen during their participation in the annual community based survey study conducted by the University of Mosul during the period from 6-19th September 2002. In addition, 45 subjects were also chosen from Al-Waffa Clinic, Ibn-Sena Hospital who were attending the clinic for checking. All these apparently healthy volunteers were chosen after excluding diabetes mellitus.

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and any other disease that may lead to proteinuria.

2. Diabetic group (Group 2): This group included 400 patients (256 females, 144 males) age range 8-87 years, 48.9 ± 14.0 years and body weight 27-127 Kg, 73.4 ± 15.9 Kg. They were known to be type 1 diabetics (48 patients) or type 2 (352 patients). They were attending Al-Waafa Diabetic Clinic, Ibn-Sena Hospital in Mosul city during a period of six months from 1st August 2002 to 31st January 2003.

This group was classified according to the degree of proteinuria into: normoproteinurics or normalalbuminurics with PCI or ACR ≤ 95th percentile confidence limit of the control group 1; and proteinurics or albuminurics with PCI or ACR > 95th percentile confidence limit of the control group 1. Microalbuminurics was considered to be present in diabetics having ACR higher than the cut-off in the control group 1 and less than 300 mg/dl, while macroalbuminurics was defined when ACR is more than 300 mg/dl.

From each subject, a morning urine sample was voided into a clean plastic container and blood sample was obtained in the fasting state between 8-10 am into fluoride-oxalate container for the measurement of plasma glucose. Urine protein was measured by two methods using in-house reagents. A turbidimetric method utilizing sulphasalicylic acid (SSA) was used that is based on the reaction of SSA with protein forming turbidity, the intensity of which varies with different protein concentrations. A dye binding method utilizing pyrogallol red (PGR) was also used where PGR forms a complex with protein resulting in a shift of the absorbance spectrum. Urine microalbumin was measured by immunoturbidimetric assay (IT) using kit from Dialab (Belgium). The turbidity is caused by the formation of antigen-antibody insoluble complexes which is accelerated and enhanced by polyethylene glycol. Urine creatinine was measured by Jaffe end point method and the ratio of albumin or protein to creatinine concentrations was calculated and expressed as ACR and PCI respectively. Plasmas glucose was estimated by glucose-oxidase-peroxidase method, using a kit supplied by Randox Ltd (England).

Analysis for urine protein and creatinine was done for all 400 patients and 145 controls; and for microalbumin was done for only 118 patients and 70 controls (because of limited availability of MA kit).

The validity (performance) indicators include: 1. Sensitivity and Specificity, 2. Predictive values, and 3. Accuracy ratio (efficiency).

Statistical methods: The statistical methods used included standard statistical methods of the mean, standard deviation (SD), standard error (SE), and range. Unpaired student Z-test was used to compare the results among subjects in the different groups. The difference between observations was considered significant at P<0.05. Linear regression analysis was also performed between ACR and PCI in the various groups. The prevalence rate of MA or proteinuria was calculated as:

Number of subjects with abnormal ACR or PCI/population size X 100.

RESULTS

The Biochemical parameters in random urine specimens, reflected as urine protein or albumin concentrations and as ratios with creatinine concentration, expressed as PCI or ACR are presented in (Table 1). In comparison with control group, the mean ± SE of PCI in the diabetic group 2 was 391.2 ± 30 mg/dl (Vs 86.0 ± 3.6 mg/dl in the controls) using SSA method, and 294.6 ± 40.4 mg/dl (Vs 73.2 ± 3.6 mg/dl in the controls) using PGR method. The ACR in the diabetic group 2 was 81.4 ± 14.1 mg/dl (Vs 18.8 ± 14.4 mg/dl in the controls) when IT method was used for albumin assay. When the three different methods were compared, a highly significant difference (P < 0.001) was noticed between groups 1 and 2 regarding urine protein, albumin, creatinine, PCI and ACR values.
Table (1): Urine biochemical characteristics in control group 1 and diabetic group 2, using sulphydryl-acidic (SSA), pyrogallal red (PGR) and immunometabolic (IT) methods. Data including protein:creatinine index (PCI) and fasting plasma glucose (FPG) are presented as mean ± SE.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (Controls)</th>
<th>Group 2 (Diabetics)</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- SSA</td>
<td>145</td>
<td>84 ± 3.5</td>
<td>10-250</td>
<td>400</td>
</tr>
<tr>
<td>- PGR</td>
<td>145</td>
<td>58 ± 3.7</td>
<td>10-240</td>
<td>400</td>
</tr>
<tr>
<td>- IT</td>
<td>70</td>
<td>15 ± 0.99</td>
<td>3-44</td>
<td>110</td>
</tr>
<tr>
<td>Creatinine (g/L)</td>
<td>145</td>
<td>0.95 ± 0.04</td>
<td>0.28-2.8</td>
<td>400</td>
</tr>
<tr>
<td>PCI (mg/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- SSA</td>
<td>145</td>
<td>80.0 ± 3.6</td>
<td>16-165</td>
<td>400</td>
</tr>
<tr>
<td>- PGR</td>
<td>145</td>
<td>73.2 ± 3.6</td>
<td>16-201</td>
<td>400</td>
</tr>
<tr>
<td>- IT</td>
<td>70</td>
<td>18.3 ± 1.4</td>
<td>4-51</td>
<td>110</td>
</tr>
<tr>
<td>FPG (mmol/L)</td>
<td>145</td>
<td>4.7 ± 0.17</td>
<td>2.3-6.4</td>
<td>400</td>
</tr>
</tbody>
</table>

Reference Ranges for Random Urine PCI and ACI

The frequency distribution of random urine PCI and ACI in control group by the three methods are shown in (Figure 1, 2 and 3) respectively. The pattern of distribution of PCI and ACI was log normal. Following log transformation of the data, they showed a minimal negative skewness of -0.53, -0.17 and -0.16 using SSA, PGR and IT methods respectively. The reference range of PCI using SSA method was calculated by multiplying the log SD by 2, then subtracting and adding the value to the log mean (both log mean and log SD were obtained after log transformation of the data). The antilog of the results gives the reference range (20-235 mg/g). In the same manner, the log mean ± 2 log SD of PCI using PGR was 1.70 ± 2 (0.264) and ACI using IT method was 1.189 ± 2 (0.282). The antilog of the final results was then calculated and they were 18-205 mg/g for PCI by PGR and 4-55 mg/g for ACI by IT method.

![Figure 1](1): Frequency distribution of random urine PCI, misasured by sulphydryl-acidic acid method, in the control group (group 1), (data are presented after log transformation).

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Figure (2): Frequency distribution of random urine PCI, measured by pyrogallol red method, in the control group (group 1). (data are presented after log transformation).

Figure (3): Frequency distribution of random urine ACI, measured by immunoturbidimetric method, in the control group (group 1). (data are presented after log transformation).

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Table (2): Prevalence of proteinuria and microalbuminuria in diabetics using sulphosalicylic acid (SSA), pyrogallol red (PGR) and immunoturbidimetric (IT) methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Normoalbuminuric</th>
<th>Proteinuric</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSA 400</td>
<td>279 13%</td>
<td>70 121%</td>
</tr>
<tr>
<td>PGR 400</td>
<td>266 65%</td>
<td>65 140%</td>
</tr>
<tr>
<td>IT 112</td>
<td>82 73%</td>
<td>30 27%</td>
</tr>
</tbody>
</table>

Prevalence of Proteinuria and Microalbuminuria in Diabetic Patients

Table 2 shows the prevalence of proteinuria and MA among diabetics measured by the three methods. When SSA method was used, 279 diabetics (70%) had normal protein excretion with PGI < 235 mg/dl while 121 diabetics (30%), (15% type 1, 29% type 2), had increased excretion with PGI > 235 mg/dl. Using PGR method, 266 diabetics (85%) had normal urine protein excretion, while 140 diabetics (35%), (40% type 1, 34% type 2), showed increased excretion with PGI>205 mg/dl. One hundred eighty diabetic without severe proteinuria were assessed by IT method for screen for MA. Of these, 6 diabetics had overt proteinuria as indicated by ACI > 300 mg/dl and were not further included in the evaluation using IT assay. The remaining 112 diabetics were analyzed for the presence of MA. Of these, 82 diabetics (73%) had normal urine albumin (ACI < 65 mg/dl) and 30 diabetics (27%), (21% type 1, 29% type 2), had MA with ACI 55-300 mg/dl.

Validity of Sulphosalicylic Acid and Pyrogallol Red Methods in Measuring Proteinuria as Alternatives to Immunoturbidimetric Method for Measuring Microalbuminuria.

The performance of SSA and PGR methods, as screening tests for proteinuria, was evaluated. The four folds contingency table was used for this evaluation of the two methods for measuring PGI as compared with IT method for measuring ACI as a gold standard. The cut-off point of ACI of 55 mg/dl and PGI 235 mg/dl (by SSA method) and 205 mg/dl (by PGR method) were used for screening MA.

Thirty diabetics were microalbuminuric with ACI between 55-300 mg/dl and considered as positive, and 82 diabetics had ACI < 55 mg/dl and considered as negatives. Twenty four diabetics (by SSA and twenty eight diabetics (by PGR) had PGI values higher than the cut off point and they represented (true positives), while other 6 diabetics (by SSA) and 2 diabetics (by PGR) had PGI values lower than the cut off level and thus considered as (false negatives). On the other hand, 78 diabetics (by SSA) and 79 diabetics (by PGR) had PGI values lower than the cut off point and considered as (true negatives). The remaining 4 diabetics (by SSA) and 3 diabetics (by PGR) had elevated PGI and represented (false positives). The sensitivity, specificity, positive predictive value, negative predictive value and accuracy rates were 93%, 90%, 95%, 95% and 95.5% respectively for PGR; and 90%, 95.1%, 86%, 93% and 91% respectively for SSA.

Comparison between PGI measured by PGR or SSA methods and ACI measured by IT method was also done using regression analysis. The correlation between the results from control group 1 was (r = 0.55, P < 0.001) for SSA and (r = 0.51, P < 0.001) for PGR method. In the diabetic group 2, the correlation was (r = 0.87, P < 0.001) for SSA and (r = 0.83, P < 0.001) for PGR method. The overall correlation in all non-diabetic and diabetic groups 1 and 2 was (r = 0.88, P < 0.001) for SSA and (r = 0.85, P < 0.001) for PGR method. From these data, values for urine PGI, as assessed by SSA or PGR, can be predictive of ACI by multiplying the PGI values by 0.375 for PGR method and by 0.39 for SSA method.

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DISCUSSION

In the current study, the frequency distribution of PCI and ACI in the control group was log normal, in comparison with others, Tanirwaki et al. reported also a log normal distribution while others reported normal Gaussian pattern. This variation may be due to the difference in the sampling methods or population size. The reference range for random urine P i was 20-235 mg/l using SSA and 18-205 mg/l using PQR. When compared with others, comparable data were obtained. Al-Jawadi and Mula-Abed in another study reported a reference range of < 190 mg/l in adults; up to 372 mg/l in pregnancy as was observed by Gupta and Gupta. For ACI, the reference range in this study was 4-55 mg/l. The cut-off level of 55 mg/l is important for deciding those subjects with increased albumin excretion or MA. Nelson et al. in a population-based study among pima Indians with type 2 diabetes showed also a cut-off of 30-300 mg/l. The same cut-off range of 30-299 mg/l for MA and ≥ 300 mg/l for macroalbuminuria was defined by Nomiyama et al. The variation in these cut-offs may be due to the effect of many factors including methodological difference for protein estimation. Variation in the amount of creatinine excreted in urine depending on muscle mass that may be affected by age, gender, ethnicity and diet may also play a part.

In this study, the prevalence of proteinuria in Iraqi diabetics was 30% using SSA and 35% using PQR and the prevalence of MA was 27%. Different prevalence rates of proteinuria and MA were noticed in different studies with a range of 13.1-49.3% for MA and 15.3-34% for proteinuria. Many factors may explain the variation in the prevalence rates when interpretation definition of MA (cut-off values) and method of urine collection (random or timed). The methodology of protein and albumin assays and model of expression of urine protein or albumin (excretion rate, concentration or its ratio to creatinine), size of study population and ethnic background are also contributing.

As far as the validity indicators of the different analytical methods are concerned, the positivity criteria were determined for MA by IT as an ACI 55-300 mg/l and for PCI as more than 235 mg/l by SSA as and more than 205 mg/l by PQR. Accordingly, the sensitivity, specificity, PPV, NPV and accuracy rates were 93%, 90%, 90%, 80% and 95.5% respectively for PQR, and 80%, 95.1%, 80%, 93% and 91% respectively for SSA. This means that the PQR method is higher ability than SSA method to give positive result for proteinuria in diabetic patients who truly have MA. Both PQR and SSA methods have nearly equal capacity to detect negative result for proteinuria in those patients who are normoalbuminuric. The findings mean that the PQR misclassifies 7% SSA 20% of microalbuminuric diabetic patients as being normoalbuminuric. However, PQR labels only 4% and SSA 5% of subjects with proteinuria when they normoalbuminuric. The high true negative rate of the test among normoalbuminuric would give a high negative predictive value which indicates that the probability of subject to be normoalbuminuric when the result of the test is negative is 98% for PQR and 93% for SSA. The lower true positive rate would make the probability of subject to be microalbuminuric using the test, when its result is positive, is 90% for PQR and 86% for SSA. High accuracy rates of 95.5% for PQR and 91% for SSA were obtained. A highly significant correlation was also observed between PCI and ACI results (r = 0.85 using PQR, and r = 0.86 using SSA). Precision of ACI and PCI results can be multiplied by the value by 0.33 using PQR and SSA methods.

Studies conducted concerning analyte comparison between immunochromatographic methods for MA and other methods for protein are lacking. To the best of my knowledge the study by Phillipou et al. represents comparable work between immunonephelometry for MA and PQR proteinuria. In their study which included diabetics, the positivity criteria of MA by IT was 96.7% and 95.3%, respectively with correlation of 0.93 and concur constant of 0.5. These values were comparable to the values in the current study. In addition, the SSA and PQR cheap methods that can provide acceptable results at only 0.3% of the cost for IT, and the reagents being commonly available can be prepared in large batches. The criteria of practicability will add to the criteria of reliability, particularly PQR offering acceptable alternatives immunochromatographic assay for urine MA.

In conclusion: the reference ranges for urine PCI and ACI are in agreement with the ranges reported by others. The PQR method shows higher validity index than SSA for measuring protein compared to IT method for measuring MA.

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Both methods show significant correlation with IT and with prediction of ACI can be made from PGI value. It is recommended to screen for proteinuria using these methods, as they are more available and less expensive. The IT method can be reserved for specimens with mild proteinuria to screen for MA.

REFERENCES


