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

Waad-Allah S., Mula-Abed, Alyaa R. Al-Kateeb

Department of Biochemistry, College of Medicine
University of Mosul

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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 sulphosalicylic acid (SSA) and pyrogallol red (PGR) methods for measuring urine protein as alternatives to immunoturbidimetric (IT) method for measuring urine microalbumin, in Iraqi diabetics with or without microalbuminuria (MA) or/and proteinuria.

Subjects and Methods: Random urine and fasting blood specimens were collected from 400 diabetics (256 females, 144 males) aged 8-87 years, including 48 type 1 and 352 type 2 diabetics. They were attending Al-Waffa Diabetic Clinic in Mosul during 6 months from 1st August 2002 to 31st January 2003. A control group of 145 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 Z-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 80%, 95.1%, 86%, 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, sulphosalicylic acid, pyrogallol red.

الخلاصـــة

الأهداف: ١. إيجاد المجال المرجعي لنسبة البروتين: كرياتنين (ب/ك) والألبومين: كرياتنين (أ/ك) لعينات إدرار عشوائية لأشخاص طبيعيين ، باستخدام طرق تحليلية مختلفة . ٢. مقارنة طريقتي حامض سالسيلك الكبريت والبايروكالول الأحمر لقياس البيلة البروتينية مع طريقة العكرة المناعية لقياس البيلة الزلالية الدقيقة .

طرق إجراء البحث والمشاركون: تم جمع عينات إدرار عشوائية وعينات دم في حالة الصوم من ٤٠٠ شخصاً مصاباً بداء السكري (١٤٤ ذكر ، ٢٥٦ أنثى) تراوحت أعمارهم بين ٨-٨٧ سنة (٤٨ سكرياً من النوع الأول ، ٣٥٢ سكرياً من النوع الثاني) . تم اختيار هؤلاء المرضى من عيادة الوفاء للسكري في الموصل خلال فترة ستة أشهر من ٢٠٠٢/٨/١ ولغاية ٢٠٠٣/١/٣١ . المجموعة الثانية من العينة تكونت من ١٤٥ شخصاً طبيعياً

(١٠٨ أنثى ، ٣٧ ذكر) تراوحت أعمارهم بين ١٥-٧٢ سنة . الفحوصات التي أجريت على كلتا المجموعتين تضمنت قياس كمية البروتين في الإدرار باستخدام طريقتي حامض سالسيلك الكبريت وطريقة البايروكالول الأحمر وكمية الكرياتنين في الإدرار لكافة المشاركين . أما قياس البيلة الزلالية الدقيقة بطريقة العكرة المناعية فقد أجريت لـ ١١٢ مريضاً و ٧٠ شخصاً طبيعياً ، كما تم قياس تركيز سكر العنب في البلازما لكافة المشاركين .

النتائج: أظهر توزيع نسب (ب/ك) و(أ/ك) عند الأشخاص غير السكريين توزيعاً طبيعياً لوغاريتمياً ، لذا تم احتساب المجال المرجعي بعد تحويل النتائج إلى قيم لوغاريتمية ، وقد كان ٢٠-٢٣٥ ملغم/غم بطريقة حامض سالسيلك الكبريت و١٨-٢٠٥ ملغم/غم بطريقة البايروكالول الأحمر ، أما بطريقة العكرة المناعية فقد كان المجال المرجعي ٤-٥٥ ملغم/غم لتكون هذه القيم الحد الفاصل في تشخيص البيلة البروتينية والبيلة الزلالية الدقيقة . كان مدى انتشار البيلة البروتينية لمرضى السكري ٣٠% بطريقة حامض سالسيلك الكبريت و٣٥% بطريقة البايروكالول الأحمر أما مدى انتشار البيلة الزلالية الدقيقة فقد كان ٢٧% . تمت مقارنة طرق حامض سالسيلك الكبريت والبايروكالول الأحمر مع طريقة العكرة المناعية ، كانت الحساسية والخصوصية والقيمة المتنبئة الموجبة والسالبة والدقة لطريقة البايروكالول الأحمر ٩٣% و٩٦% و٩٠% و٩٨% و٩٥,٥% بالتعاقب . أما طريقة حامض سالسيلك الكبريت فقد كانت القيم ٨٠% و٩٥,١% و٨٦% و٩٣% و٩١% بالتعاقب مع ترابط معتدا عاليا (ب < ٠,٠٠١) بين قيم (أ/ك) و(ب/ك) بطريقة البايروكالول الأحمر (ر = ٠,٨٥) وطريقة حامض سالسيلك الكبريت (ر = ٠,٨٧) . كما لوحظ إمكانية حساب قيمة (أ/ك) من قيمة (ب/ك) بضرب قيمة (ب/ك) بـ ٠,٣٧٥ لطريقة البايروكالول الأحمر و٠,٣٩ لطريقة حامض سالسيلك الكبريت .

الاستنتاج: إن البيلة البروتينية والبيلة الزلالية الدقيقة منتشرة بين السكريين العراقيين وأن الطرق السهلة والمتوفرة خاصة طريقة البايروكالول الأحمر كان أداءها مقبول ويمكن استخدامها بشكل روتيني خلال العناية بالمرضى السكريين مع التوصية بضرورة قياس نسبة (ب/ك) في الإدرار لكل شخص سكري خلال زيارته المنتظمة للعيادات المخصصة للداء السكري .

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[1]. It is so common 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[2].

Measurement of urine albumin excretion is used for the early diagnosis of diabetic nephropathy and for monitoring the effectiveness of treatment[3]. 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) have been proposed[4]. The random urine sampling is simpler and easier than 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[5,6].

Many methods for measuring protein or albumin in urine have been reported. However, none is completely satisfactory, and all assays suffer from standardization problem[7]. The currently available methods include dip stick testing[8] as well as qualitative and quantitative techniques. For quantitation, different approaches are available including turbidimetric[9], dye-binding[10] and immunochemical methods[11]

The aims of the current study were: 1. To derive a reference range for random urine PCI and ACI in apparently healthy subjects, using different analytical methods; and 2. To assess the validity and performance indicators of dye binding or turbidimetric methods for measuring urine protein in comparison with immunochemical method for measuring urine albumin in diabetic subjects with or without MA or/and proteinuria.

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-Hmedaat 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 6th-18th 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

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-Waffa 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 normoalbuminurics with PCI or ACI ≤ 95th percentile confidence limit of the control group 1; and proteinurics or albuminurics with PCI or ACI > 95th percentile confidence limit of the control group 1. Microalbuminuria was considered to be present in diabetics having ACI higher than the cut-off in the control group 1 and less than 300 mg/g, while macroalbuminuria was defined when ACI is more than 300 mg/g[12].

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 sulphosalicylic 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[19]. 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 [10]. 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 [13]. Urine creatinine was measured by Jaffe end point method[14] and the ratio of albumin or protein to creatinine concentrations was calculated and expressed as ACI and PCI respectively. Plasma glucose was estimated by glucose-oxidase-peroxidase method, using a kit supplied by Randox Ltd (England)[15]. 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)[16].

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 $p<0.05$[17]. Linear regression analysis was also performed between ACI and PCI in the various groups. The prevalence rate of MA or roteinuria was calculated as:

Number of subjects with abnormal ACI 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 ACI are presented in (Table 1). In comparison with control group, the mean ± SE of PCI in the diabetic group 2 was 391.2 ± 43.6 mg/g (Vs 80.0 ± 3.6 mg/g in the controls) using SSA method, and 364.6 ± 40.4 mg/g (Vs 73.2 ± 3.6 mg/g in the controls) using PGR method. The ACI in the diabetic group 2 was 81.4 ± 14.1 mg/g (Vs 18.8 ± 1.4 mg/g 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 ACI values.

Table (1): *Urine biochemical characteristics in control group 1 and diabetic group 2, using sulphosalicylic acid (SSA), pyrogallol red (PGR) and immunoturbidimetric (IT) methods. Data including protein:creatinine index (PCI) and fasting plasma glucose (FPG) are presented as mean ± SE.*

Characteristic	Group 1 (Controls)			Group 2 (Diabetics)			Z	P
	n	Mean ± SE	Range	N	Mean ± SE	Range		
Protein (mg/L)								
- SSA	145	64 ± 3.5	10-250	400	225 ± 20.6	10-3300	- 7.7	<0.001
- PGR	145	58 ± 3.7	10-240	400	219 ± 19.7	10-3900	- 8.1	<0.001
- IT	70	15 ± 0.99	3-44	118	51 ± 7.4	5-480	- 4.9	<0.001
Creatinine (g/L)	145	0.95 ± 0.04	0.28-2.8	400	0.75 ± 0.02	0.08-4.3	- 5.7	<0.001
PCI (mg/g)								
- SSA	145	80.0 ± 3.6	16-185	400	391.2 ±43.6	12-7857	- 7.3	<0.001
- PGR	145	73.2 ± 3.6	16-201	400	364.6 ±40.4	7-9285	- 7.3	<0.001
- IT	70	18.8 ± 1.4	4-51	118	81.4 ± 14.1	3-1067	- 5.7	<0.001
FPG (mmol/L)	145	4.7 ± 0.11	2.3-9.4	400	10.7 ± 0.23	2.7-25.2	- 14.7	<0.001

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 1.79 ± 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.

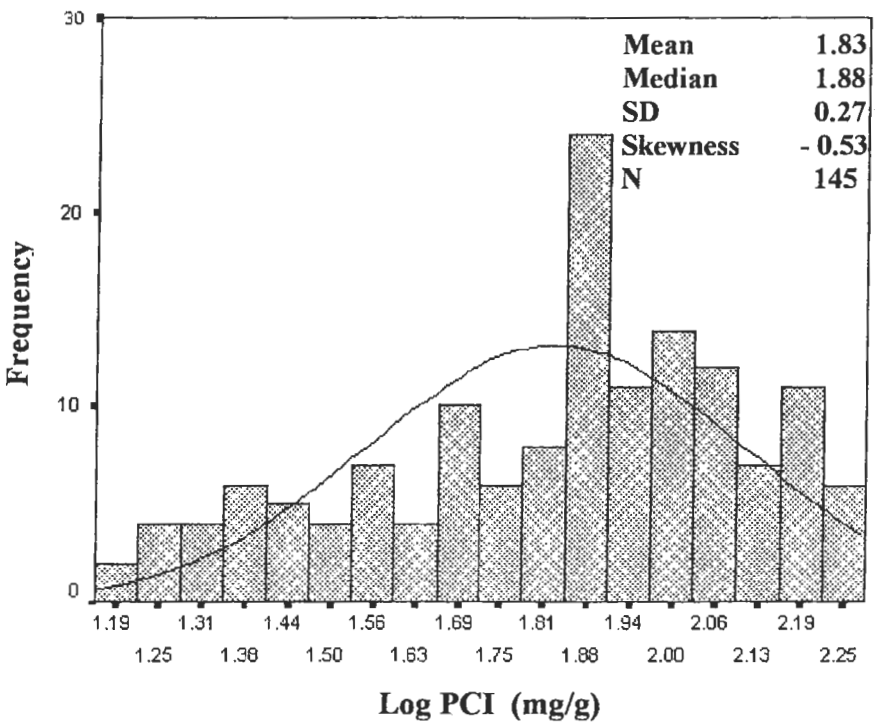

Figure (1): *Frequency distribution of random urine PCI, measured by sulphosalicylic acid method, in the control group (group 1), (data are presented after log transformation).*

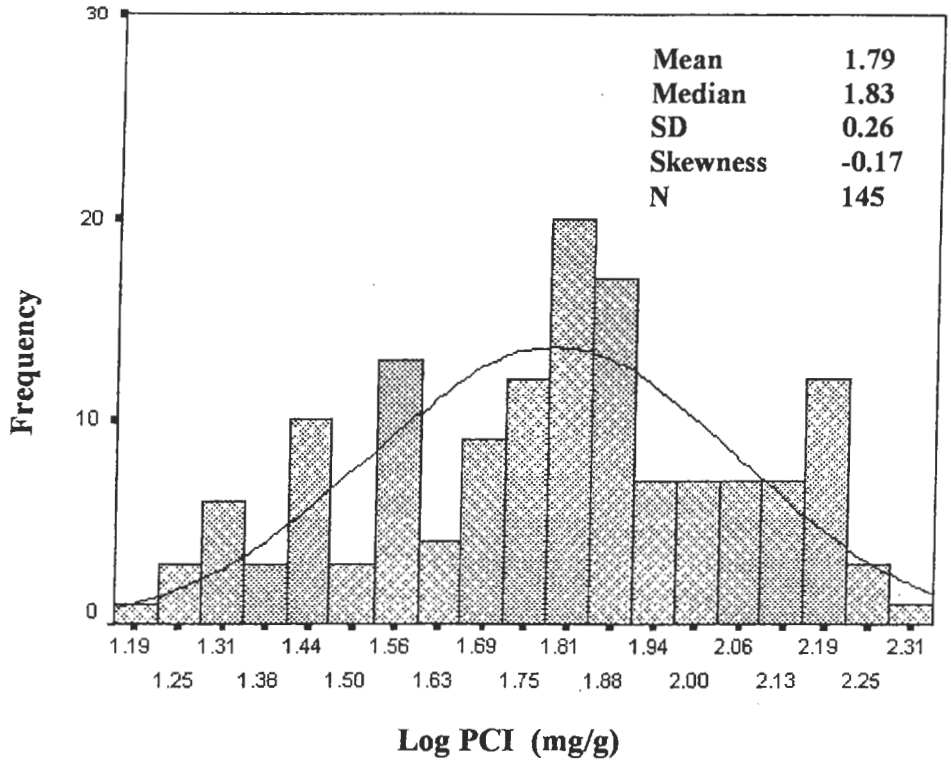

Figure (2): *Frequency distribution of random urine PCI, measured by pyrogallol red method, in the control group (group 1), (data are presented after log transformation).*

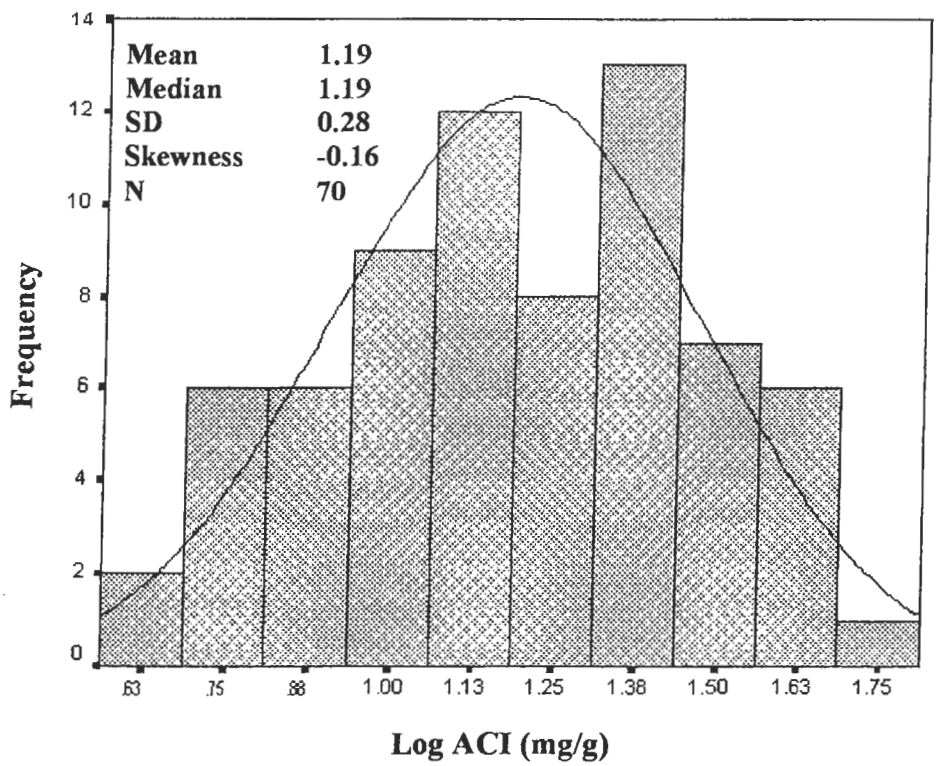

Figure (3): *Frequency distribution of random urine ACI, measured by immunoturbidimetric method, in the control group (group 1), (data are presented after log transformation).*

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

Method	N	Normoproteinuric		Proteinuric	
		n	%	N	%
SSA	400	279	70	121	30
PGR	400	260	65	140	35
IT	112	Normoalbuminuric		Microalbuminuric	
		N	%	N	%
		82	73	30	27

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 PCI ≤ 235 mg/g while 121 diabetics (30%), (35% type 1, 29% type 2), had increased excretion with PCI > 235 mg/g. Using PGR method, 260 diabetics (65%) had normal urine protein excretion, while 140 diabetics (35%), (40% type 1, 34% type 2), showed increased excretion with PCI>205 mg/g. One hundred eighteen diabetics without severe proteinuria were assessed by IT method to screen for MA. Of these, 6 diabetics had overt proteinuria as indicated by ACI > 300 mg/g 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 ≤ 55 mg/g) and 30 diabetics (27%), (21% type 1, 28% type 2), had MA with ACI 55-300 mg/g.

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 PCI as compared with IT method for measuring ACI as a gold standard. The cut-off point of ACI of 55 mg/g and PCI 235 mg/g (by SSA method) and 205 mg/g (by PGR method) were used for comparison.

Thirty diabetics were microalbuminuric with ACI between 55-300 mg/g and considered as positives, and 82 diabetics had ACI < 55 mg/g and considered as negatives. Twenty four diabetics (by SSA) and twenty eight diabetics (by PGR) had PCI values higher than the cut off point and they represented (true positives), while other 6 diabetics (by SSA) and 2 diabetics (by PGR) had PCI 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 PCI 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 PCI and represented (false positives). The sensitivity, specificity, positive predictive value, negative predictive value and accuracy rates were 93%, 96%, 90%, 98% and 95.5% respectively for PGR; and 80%, 95.1%, 86%, 93% and 91% respectively for SSA.

Comparison between PCI measured by PGR or SSA methods and ACI measured by IT method was also done using regression analysis. The correlation between the results in control group 1 was ($r= 0.50$, $P< 0.001$) 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 PCI, as assessed by SSA or PGR, can be predictive of ACI by multiplying the PCI values by 0.375 for PGR method and by 0.39 3 for SSA method.

DISCUSSION

In the current study, the frequency distribution of PCI and ACI in the control group was log normal. In comparison with others, Taniwaki *et al.*[18] reported also a log normal distribution while others reported normal Gaussian pattern[19,20]. This variation may be due to the difference in the sampling methods or population size.

The reference range for random urine PCI was 20-235 mg/g using SSA and 18-205 mg/g using PGR. When compared with others, comparable data were obtained. Al-Jawadi and Mula-Abed[6] in another study reported a reference range of ≤ 190 mg/g in adults,up to 37-247 mg/g as was observed by Gupta and Gupta[21]. For ACI, the reference range in this study was 4-55 mg/g. The cut-off level of 55 mg/g is important for deciding those subjects with increased albumin excretion or MA. Nelson *et al.*[22] in a population-based study among pima Indians with type 2 diabetes showed also a cut-off of 30-300 mg/g. The same cut-off range of 30-299 mg/g for MA and ≥ 300 mg/g for macroalbuminuria was defined by Nomiyama *et al.*[23] . 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[24].

In this study, the prevalence of proteinuria in Iraqi diabetics was 30% using SSA and 35% using PGR 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[23,25,,26,27,28]. Many factors may explain the variation in the prevalence rates which include the 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[29].

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/g and for PCI as more than 235 mg/g by SSA as and more than 205 mg/g by PGR. Accordingly, the sensitivity, specificity, PPV, NPV and accuracy rates were 93%, 96%, 90%, 98% and 95.5% respectively for PGR; and 80%, 95.1%, 86%, 93% and 91% respectively SSA. This means that the PGR method higher ability than SSA method to giv positive result for proteinuria in diabe who truly have MA. Both PGR and S methods have nearly equal capacity to g negative result for proteinuria in those are normoalbuminuric. The findings mean that the PGR misclassifies 7% SSA 20% of microalbuminuric diabetics being normoproteinuric. However, P labels only 4% and SSA 5% of subjects proteinurics when they normoalbuminurics. The high true nega rate of the test among normoalbuminu would give a high negative predictive va which indicates that the probability of subject to be normoalbuminuric when result of the test is negative is 98% for P and 93% for SSA. The lower true posi rate would make the probability of subject to be microalbuminuric using test, when its result is positive, is 90% PGR and 86% for SSA. High accur rates of 95.5% for PGR and 91% for S were obtained. A highly signific correlation was also observed between and PCI results (r = 0.85 using PGR, a = 0.88 using SSA). Prediction of ACI f PCI value can be made by multiplying value by 0.33 using PGR and S methods.

Studies conducted concerning analyt comparison between immunochem methods for MA and other methods protein are lacking. To the best of available knowledge, the st by Phillipou *et al.*[30] represents comparable work betw immunonephlometry for MA and PGR proteinuria. In their study which inclu 179 diabetics, the sensitivity and specifi of PGR was 96.7% and 95.3%, respectiv with correlation of 0.93 and convers constant of 0.5. These values comparable to the values in the curr study. In addition, the SSA and PGR cheap methods that can provide accepta results at only 0.3 % of the cost for IT, v the reagents being commonly available can be prepared in large batches. Th criteria of practicability will add to criteria of reliability, particularly PGR offering acceptable alternatives immunochemical assay for urine MA.

In conclusion: the reference ranges urine PCI and ACI are in agreement v the ranges reported by others. The P method shows higher validity indicat than SSA for measuring protein compared to IT method for measuring M

Both methods show significant correlation with IT and with prediction of ACI can be made from PCI 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 search for MA.

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