Screening for microalbuminuria by use of microproteinuria

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تحري البيلة المكروألبومينيَّة باستخدام طريقة البيلة المكروبروتينية

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الخلاصة: هَدَفَ الباحثون إلى ابتكار طريقة عملية يعول عليها وتكون زهيدة الكلفة، لتقييم المراحل الأولى للإصابة بالاختلال الكلوي لدى مرضى السكري، وذلك لاستخدامها في الأشخاص الـمُخْتَطَرين اختطاراً عالياً في البلدان المحدودة الموارد. وقام الباحثون أولاً بتقييم طريقة تعتمد على قياس العَكَر باستخدام حمض ثلاثي كلور أسيتيك لتحري البيلة المكروبروتينية في مرضى السكري. وتمت مقارنة هذه الطريقة بطريقة عكرية مناعية لكشف البيلة المكروألبومينية. وتم إجراء كلتا الطريقتين ضمن الحدود التي يسمح بها مبدأ اللاتيقُّن تأسيساً على التباين بين الأفراد وضمن الأفراد. وقد تبين أن نسبةً مقدارُها 3.0 غ/مول من الألبومين/الكرياتينين في البول باعتبارها مشعراً تشخيصياً للبيلة المكروألبومينية، تترابط ترابطاً وثيقاً بمقدار فَيْصَل cut-off يبلغ 24 مغ/ل من البيلة المكروبروتينية. وكانت الحساسية والنوعية السريرية لطريقة حمض ثلاثي الكلور أسيتيك المحددة مقابل هذه النسبة هي 86٪ و90٪ على التوالي. ويرى الباحثون أن مُعوَّلية طريقة حمض ثلاثي الكلور أسيتيك ومَيْسوريَّتها تجعلانها مناسبة لكشف المراحل الباكرة من الإصابة بالتلف الكلوي، مؤكِّدينَ على أهميَّتها بشكل خاص في تَقَصِّي المرض لدى الأشخاص الـمُخْتَطَرين اختطاراً عالياً في البلدان المحدودة الموارد.

ABSTRACT We aimed to develop a reliable, low cost method to assess the early stages of renal impairment in diabetes, for use in high-risk populations in countries with limited resources. We evaluated a trichloroacetic acid (TCA) turbidimetric method for microproteinuria screening in patients with diabetes. The method was compared with an immunoturbidimetric procedure for the detection of microalbumuniuria. Both methods performed within limits of allowable uncertainty based on inter- and intra-individual variation. A urinary albumin/creatinine ratio of 3.0 g/mol, assumed as diagnostic of microalbuminuria, was found to correlate with a cut-off value of 24 mg/L for microproteinuria. The clinical sensitivity and specificity of the TCA method determined against this ratio were 86% and 90% respectively. The reliability and practicability of the TCA method renders it suitable for the detection of early stage renal damage, with emphasis on screening high-risk populations in countries with limited resources.

Dépistage de la microalbuminurie au moyen de la microprotéinurie

RÉSUMÉ Notre objectif était de mettre au point une méthode, fiable et peu coûteuse pour évaluer les premiers stades de l'insuffisance rénale dans le diabète afin de l'utiliser dans les populations à haut risque des pays qui ont des ressources limitées. Nous avons évalué une méthode turbidimétrique utilisant l'acide trichloroacétique pour le dépistage de la microprotéinurie chez des patients diabétiques. Celle-ci a été comparée à la méthode immunoturbidimétrique pour le dépistage de la microalbuminurie. Les deux méthodes ont produit des résultats se situant dans les limites de l'incertitude permise sur la base de la variation inter- et intra-individuelle. On a trouvé une corrélation entre un rapport albumine/créatinine urinaire de 3,0 g/mol, sur la base duquel est diagnostiquée une microalbuminurie, et une valeur seuil de 24 mg/L pour la microprotéinurie. La sensibilité et la spécificité cliniques de la méthode à l'acide trichloroacétique déterminées en fonction de ce rapport étaient de 86 % et 90 % respectivement. La fiabilité et la praticabilité de la méthode à l'acide trichloroacétique la rendent appropriée pour le dépistage d'une atteinte rénale précoce, en particulier le dépistage dans les populations à haut risque des pays qui ont des ressources limitées.

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Introduction

There is currently a global epidemic of type 2 diabetes. At present an estimated 150 million people worldwide have diabetes, a figure that is predicted to rise to 300 million by 2025 [*1*]. Surveys have indicated that while diabetes is widely recognized in North American and western European countries, there is also a high prevalence in the developing world and type 2 diabetes has reached epidemic proportions in many such countries [*2*]. Hence diabetes is a major concern for the developing as well as more developed countries.

Diabetes is the most common single cause of end-stage renal disease (ESRD) in the United States (US) and Europe [*3*]. About 20%–30% of patients with type 1 or 2 diabetes develop evidence of nephropathy. The onset of diabetic nephropathy can be significantly ameliorated by intervention, instituted early in the course of the development of this complication, at a stage when microalbuminuria is present [*3*].

Microalbuminuria is one of the earliest markers of renal disease in diabetes [*4*], and is a sensitive marker for the development of diabetic nephropathy. A strong correlation has been reported between renal function, the degree of albuminuria and structural renal change [*5*]. Small increases in the urinary albumin excretion rate in patients with diabetes may be associated with subsequent development of clinical nephropathy. It has been reported that the presence of microalbuminuria increases the risk of development of diabetic nephropathy 10-fold [*4*]. Also in patients with type 2 diabetes, nephropathy is loosely associated with large vessel disease. Early intervention to reduce coronary events, control of hypertension and the introduction of angiotensin-converting enzyme inhibitors can greatly improve the prognosis [*6*]. Therefore the determination of the albumin excretion rate has found extensive use in monitoring diabetic populations for early detection of renal damage and in this context an active approach to screening for diabetic nephropathy and its management is recommended [*6*].

Despite widespread acceptance of the value of screening for microalbuminuria, there is no consensus as to the most appropriate urine sample for screening of microalbuminuria [*7*]. Various procedures have been proposed, including 24-hour urine collection, overnight collection and random urine collection (preferably early morning/first voided) [*8,9*], the latter being most convenient for the patient. Determination of the albumin/creatinine ratio in a urine sample further increases the diagnostic usefulness of random urine measurement [*7*]. However, again there is no agreement on the reference albumin/creatinine ratio cut-off. Different values have been proposed; while some studies suggest a need for age and sex discriminator values [*10*], others propose a single cut-off of 2.5 g/mol for both sexes [*11*]. In our laboratory a cut-off value of 3.0 g/mol is used to distinguish microalbuminuria from normoalbuminuria.

Many methods have been developed for the measurement of albumin in urine, nearly all based on immunoassay principles [*12–15*]. This technology, although reliable in terms of performance characteristics, includes reagents with relatively short biological half-lives, which results in greater cost. It may also require specialized instrumentation and technical skill. In this context, although assessment of renal function is of utmost importance in monitoring patients with diabetes, the standard immunoassays are unsuitable for use in some developing countries where resources may be limited.

The purpose of this study was to find a simple, cost-effective and yet reliable method to detect microalbuminuria that could be recommended to laboratories with limited resources. In this context a manual trichloroacetic acid (TCA) turbidimetric protein assay [*16*] with reliable performance specifications in the desired range was evaluated. Comparison of the TCA method for microproteinuria and an immunoassay procedure for microalbuminuria (expressed in terms of albumin/creatinine ratio) was carried out

Methods

Materials

Albumin immunoassay kits including appropriate calibrators were obtained from Randox Laboratories (Crumlin, United Kingdom). A creatinine kit based on the Jaffé reaction was also obtained from Randox Laboratories. Creatinine powder (Sigma–Aldrich cat. no. C-4255, Dorset, UK), was used for in house preparation of calibration standards. Trichloroacetic acid was obtained from BDH (Leicestershire, UK). Saline (9 g/L) for dilution of calibrator and controls was purchased commercially from Baxter (Mallusk, UK).

Instruments

Albumin and creatinine were assayed on a Cobas Fara analyser (Roche Diagnostics). Both assays were carried out according to the manufacturer's protocol. Imprecision in terms of coefficient of variation was estimated at 1.6% and 5% respectively. Bias for both assays, validated on the basis of the Bias Index Score (BIS) in the UK National External Quality Assessment Scheme (UK NEQAS), was found to be less than 50, which is indicative of appropriate performance. Urinary protein concentrations as determined by the TCA method were examined by both the Cobas Fara analyser and manually. In the manual measurement a Wallac Biochrom 4060 photometer was used to record the absorbance.

Samples

Samples sent for assessment of microalbuminuria from primary and secondary care diabetes clinics were used. Over 300 specimens, from both sexes and covering the whole spectrum of the analytical interval, were examined. Samples were stored either at room temperature or at 4 °C until analysis. The period of storage varied from overnight to over the weekend.

Calibrators and controls

Randox urinary protein calibrator (Cat. No. ST 1568) was used to calibrate the TCA method. As the concentration of this calibrator (1000 mg/L) exceeded the concentrations of interest for the linearity limit of the assay [*16*], a working calibrator (500 mg/L) was prepared by dilution in saline. Albumin calibrators were provided with the kit. Creatinine calibrator (2 mmol/L) was prepared by dissolving 0.2262 g of creatinine powder in 1 L of water. This standard was aliquoted and stored at –20 °C.

Biorad Liquicheck Urine chemistry controls low and high (cat. nos.397 and 398 respectively) were used to assess imprecision. SPS-01 human serum liquid calibrator obtained from the Supra-regional Assay Service (Protein Reference Unit) with assigned values for albumin and a mixture of globulins was used to assess bias. The latter was diluted to appropriate concentrations with saline.

Assays

The TCA assay was carried out as described by Shahangian et al. [*16*]. A solution of trichloroacetic acid (765 mmol/L;

12.5 g/100 mL) was prepared in deionized water. To 1.6 mL of control, calibrator or sample, 0.4 mL of TCA reagent was added. Control and calibrators were examined in every run. For each test a complementary blank tube was set up containing the sample and TCA reagent in the same proportions as the test. The blank underwent the same procedure as the test. All tubes were mixed and allowed to stand at room temperature for 35 minutes. After this time all blank tubes were centrifuged for 10 minutes at 2000 × *g*. The photometer was set at zero with deionized water and each test read at 420 nm against the appropriate blank (supernatant of TCA treated urine). A modification of the manual method was set up on the Roche Cobas Fara analyser using the same reagent/sample ratios, but an incubation time of 15 minutes was used without affecting the assay results.

The between assay imprecision was calculated through repeated measurements of control samples at different concentrations. It was observed that the repeatability of the test decreased with decreasing concentrations; however the maximum coefficient of variation, relating to the lowest concentration of about 20 mg/L, was estimated at about 9%. The systematic error (bias) of the method as evaluated using the SPS-01 reference material was estimated at 6%. The total uncertainty was therefore well within the allowable range of 46.1% ($P < 0.05$) [*17*].

All samples were analysed for protein, albumin and creatinine on the same day. It was observed that on days when the samples had been kept for longer periods before analysis, there was greater discordance between the protein and the albumin values. Examination of the samples concerned showed that the difference was due to the presence of bacterial contamination, the frequency of which increased with prolonged storage of samples, especially at room temperature prior to examination. Bacterial contamination does not interfere with the immunoassay for albumin [*18*], but it does interfere with the turbidimetric method used for the measurement of protein [*19*]. Therefore on the basis of this finding all samples were examined microscopically for the presence of microorganisms prior to analysis; if positive, they were excluded from the study. It was also observed that rapid transfer of samples to 4 °C for storage, and analysis on the day of receipt reduced the incidence of *in vitro* contamination to insignificant levels.

Results

The results obtained from the determination of urinary protein and albumin/creatinine ratio were compared and the diagnostic effectiveness of the TCA method was evaluated. An albumin/creatinine ratio cut-off value of equal to or greater than 3.0 g/mol was used to identify the diseased population as indicated by the presence of microalbuminuria. Table 1 shows the clinical sensitivity and specificity along with the positive and negative predictive values calculated for different cut-off values of urinary protein. It should be noted that the predictive value is valid only for the population under investigation. From the table, it can be seen that the value of 24 mg/L of urinary protein gives the most appropriate clinical sensitivity and specificity. Hence this cut-off is recommended as the value above which microalbuminuria is most likely to be present.

Based on the above delineation of diseased and non-diseased states, a receiver operator characteristic (ROC) curve was constructed as shown in Figure 1. This curve shows the performance of the urinary protein method through the entire range of decision levels.

Table 1 **Results of comparison of albumin/creatinine ratio and microproteinuria**

Urinary protein concentration (mg/L)	Sensitivity (%)	Specificity (%)	(+) predictive value (%)	(–) predictive value (%)
18.7	86.7	85.5	60	96
20.0	86.7	86.3	61	96
20.2	86.7	87.2	63	96
22.0	86.7	88.0	64	96
22.2	86.7	88.9	66	96
23.0	86.7	89.7	68	96
23.9	86.7	90.6	69	96
24.1	83.3	90.6	69	95
24.5	80.0	91.5	70	95

The predictive value of the test is calculated according to the prevalence seen in the specific population under investigation, which was estimated at 20%.

The distribution of urinary albumin results in urinary protein positive and negative groups is shown in Figure 2. One extreme outlier has been excluded. The figure shows the non-parametric nature of the distribution of results, especially in the positive population.

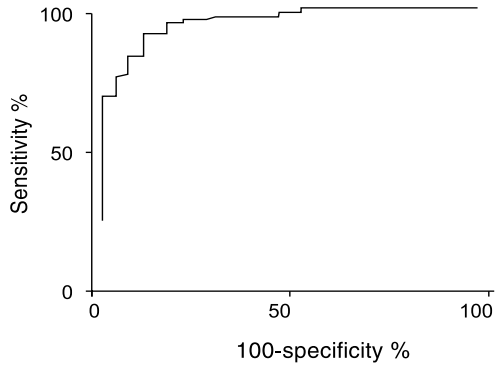

Figure 1 **Receiver operating characteristic (ROC) curve; use of urinary protein to predict an albumin/creatinine ratio of 3.0 g/mol**

Discussion and conclusions

This study was carried out in an attempt to find a simple, cost-effective and at the same time reliable method for the assessment of microalbuminuria, which could be used in laboratories at the intermediate and peripheral level in developing countries with limited resources.

The TCA method is based on the precipitation of proteins in the urine sample followed by turbidimetric quantitation. The turbidimetric methods for measurement of urinary proteins in general do not have as good quality specifications as the immunochemical methods used for the examination of urinary albumin. However, at a cut-off of 24 mg/L the clinical sensitivity of this modification of the TCA method, when

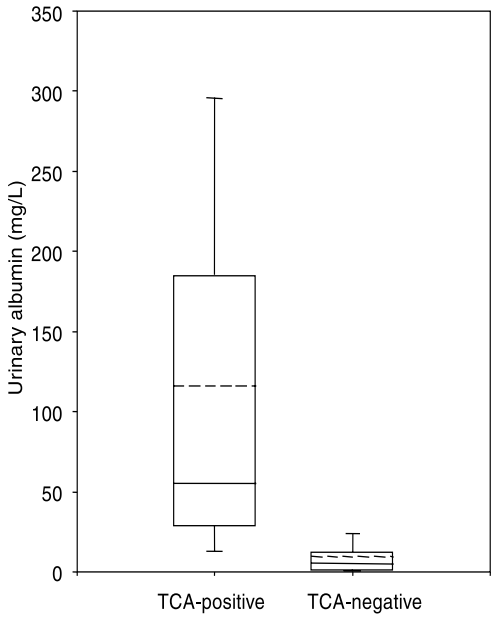

Figure 2 **Box and whisker plot of the results of the urinary protein versus urinary albumin concentrations. The dashed line indicates the mean and the solid line the median. The box covers the central 50% of results, the whiskers extend to include the 5th and the 95th centile.**

compared against the urinary albumin/creatinine ratio of 3.0 g/mol, shows that over 86% of the diseased population can be identified. At the same cut-off, the specificity is calculated at 90% bearing in mind that this is only valid in the absence of bacteriuria and contamination with microorganisms. In addition, the inexpensive and rapid nature of the test renders it affordable for regular monitoring of patients. Hence those patients falsely identified as negative are likely to be detected in further regular investigations. It should be noted that the reagent cost per test for the TCA method (~1.2 English pence per test) is approximately 30 times less than that estimated for the immunoassay determination of albumin (~36 English pence per test). Furthmore, the TCA test can be performed using simple analytical instrumentation.

The high biological variation inherent in the albumin excretion rate calls for at least 2 consecutive examinations of positive samples. We recommend that samples are analysed on the day of collection to avoid risk of contamination by microorganisms. If possible, we recommend storage at 4 °C prior to analysis. Measurement of microproteinuria on a contaminated sample will result in a false positive result. If this possibility is suspected, an additional examination should be carried on a fresh sample. The protocol recommended for the performance of the urinary protein test is shown in Figure 3.

- Samples should not be collected from patient with known urinary tract infection.
- Collect random urine sample. Preference should be given to the first sample voided in the morning.
- No preservatives should be added to samples.
- Store all samples at 4 °C until analysis.
- All samples should be examined on the day of collection.
- A patient with urinary protein $\geq$ 24 mg/L should be asked to provide a repeat sample in 2 weeks.
- In case of discordance between the 2 results a third sample should be analysed.
- Drugs taken by the patient should be noted as some can lead to false positive results [***20***].
- Patients found to have 2 urinary protein values above 24 mg/L should be selected for further investigation and management.

Figure 3 **An outline of the procedure for the measurement of urinary protein**

The TCA method as described satisfies many of the requirements for the screening of patients' samples at early stages of renal disease. It is simple, inexpensive and relatively rapid with little need for specialized skills. The reagent has a long shelf life and can be prepared in-house. In countries with limited resources we recommend the use of this method for screening of patients with diabetes at high risk of developing renal damage. Where circumstances allow, this test can be used to identify patients requiring referral for quantification of urinary albumin by an immunochemical method.

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Fluorescence microscopy for disease diagnosis and environmental monitoring

In countries with limited resources, simple, rapid and sensitive diagnostic techniques are key elements for the performance of medical and public health laboratories at all levels. Bright-field microscopy has been the primary diagnostic technique of laboratories at peripheral and district levels. Fluorescence microscopy is almost as simple to do, and most often it is more specific. In the past, the high cost of fluorescence microscopes prevented the wider application of this method. More recently, less expensive fluorescence microscopes have been developed, and accessories are now available that convert a bright-field microscope into a fluorescence microscope. This development places fluorescence microscopy in a favourable position as a method that can be used by laboratories to enhance their effectiveness at affordable cost. Laboratories should be more aware of the advantages of using fluorescence microscopy. This manual, *Fluorescence microscopy for disease diagnosis and environmental monitoring* provides information on the principles of fluorescence microscopy and practical advice on the preparation of samples for many simple applications for diagnosing disease and monitoring environmental contamination using a fluorescence microscope. The publication puts emphasis on procedures for direct, rapid identification of microorganisms causing a disease. The practical steps of indirect immunofluorescence microscopy for the diagnosis of noncommunicable diseases are also considered. The manual can be obtained from: World Health Organization Regional Office for the Eastern Mediterranean, Abdul Razzak Al Sanhouri Street, PO Box 7608, Nasr City, Cairo 11371, Egypt. Email: emr:dsa@ emro.who.int.