

Microalbuminuria measurements by two in-house ELISA methods

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Abstract

Detection of microalbuminuria is important in the management of diabetic patients since it is predictive of development of proteinuria and nephropathy. Two sensitive and specific in-house ELISAs for microalbuminuria were established and validated. One of the ELISAs was based on antigen coating while the other employed antibody coating. Recovery and linearity experiments gave acceptable results of $100 \pm 10\%$, while precision results were $<10\%$ for intra-assay and $<12\%$ for inter-assay coefficients of variation (CVs). The standard curve ranged from 10-625 $\mu\text{g/l}$, equivalent to 0.2-12.5 mg/l for urine samples diluted 1:20 fold. When the antibody coated ELISA was compared to antigen coated ELISA, a correlation of $r=0.996$ was obtained. When compared to commercial kits, the in-house ELISAs gave good correlations of $r=0.961$ versus the Boehringer Mannheim Micral Test strips and $r=0.940$ versus Ames Microalb Turbidimetry. The normal microalbumin reference ranges determined for 12h, first morning and random urine samples were 0.7-5.3 mg , 0.1-10.2 mg/l and 0.8-26.1 mg/l respectively. The normal albumin excretion rate (AER) was 1.0-7.3 $\mu\text{g/min}$ while untimed urine samples gave results of 0.1-0.9 and 0.2-1.6 mg/mmol after dividing by creatinine concentrations. The ELISAs were used to detect microalbuminuria in 338 random urine samples from diabetic patients. A high percentage 47.9% was found to be positive for microalbuminuria and 18.0% had macroalbuminuria $>25 \text{ mg/mmol}$. Thus screening for microalbuminuria together with creatinine measurements using random urine samples can be used for management of diabetic patients.

Key words; microalbuminuria, ELISA, diabetes mellitus

INTRODUCTION

Measurement of proteinuria is used together with other routine renal function blood tests to diagnose and monitor impaired renal function and nephropathy.¹⁻³ In diabetes mellitus, diabetic nephropathy is one of the chronic microvascular complications associated with persistent hyperglycaemia. Although the development of impaired renal function is chronic and progressive, its progression rate may be delayed or even reversed by early intervention treatment and through strict and good control of blood glucose levels and blood pressure.⁴⁻¹⁰ It has been reported that the risks for development of diabetic nephropathy are indeed high, in the range of 30-35% for type 2 (NIDDM) diabetic patients and up to 45% for type 1 (IDDM) patients. Thus the early identification of abnormal protein excretion in the urine prior to overt proteinuria is crucial in the management of diabetic patients. Microalbuminuria is defined as an albumin excretion rate (AER) of 20-200 $\mu\text{g/min}$ or 30-300 mg/24h and is not detected by routine

Albustix proteinuria dipstick tests. The sensitive methods available for microalbuminuria testing are immunoassays such as immunoturbidimetry or nephelometry, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).¹¹⁻¹⁵ Since colour is the end-point for the non-isotopic ELISA method, semi-quantitative immunoassay commercial dipstick tests are available." In this study, we developed two quantitative in-house ELISAs for microalbuminuria by producing polyclonal anti-human albumin antiserum, enzyme-labelled albumin and other in-house reagents. The first in-house assay developed was the antigen coated ELISA, simultaneous incubation of ligand with primary antibody and further reaction with secondary antibody labelled with enzyme. Antibody coated ELISA was the second in-house assay, which required simultaneous incubation of ligand with albumin labelled enzyme. The in-house ELISAs were optimised, validated and used for microalbuminuria measurements in normal subjects and diabetic patients.

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MATERIALS AND METHODS

REAGENTS

Phosphate buffered saline (PBS): 0.15 M PBS, pH 7.2 containing per litre 1.5g Na_2HPO_4 , 0.2 g KH_2PO_4 , 9.0 g NaCl and 0.01g thiomersal. Stable at room temperature for up to 3 months

Assay Buffer (PBS.gelatin): PBS containing 0.1% (w/v) gelatin. Stable up to 1 month at 4°C.

Coating Buffer: 0.05 M Bicarbonate buffer, pH 9.6 containing per litre 1.59 g Na, CO, and 2.94 g NaHCO_3 . Stable at 4 °C for 2 months.

Blocking Solution (PBS.gelatin): PBS containing 1.0% (w/v) gelatin. Stable up to 1 month at 4°C.

Wash Buffer (PBS.Tween): PBS containing 0.05% (v/v) Tween-20. Stable at 4°C for 2 months.

Substrate Solution: 25 mM Citrate phosphate buffer, pH 5.0 containing per litre 5.25 g citric acid and 7.1 g Na_2HPO_4 . Add just before use, 40 mg o-phenylenediamine (OPD) dihydrochloride and 60 μl H_2O_2 (30% v/v) to 100 ml solution.

Stopping Solution: 1.25 M sulphuric acid.

Standards (Human albumin (Behringwerke): A stock solution 100 mg/l was prepared and stored in small aliquots at -20°C for up to 6 months.

Quality Control (QC) Samples: Three QC pools were prepared by adding 0.2, 0.8 and 3.2 mg human albumin to 100 ml normal urine. Stored in small aliquots at -20°C.

URINE SAMPLES

Normal urine samples were collected from students and staff (n=40, males=18, females=22, age range 19-36 years) of the Faculty of Medicine, UKM. Three sets of urine samples were collected from each normal subject, random urine (RU), first morning urine (FMU) and overnight 12 hours urine. Random urine samples were also collected from diabetic patients (n=338, males=113 females=225, age range 18-73 years) from the Endocrine Clinic, UKM. These samples were also tested with Albustik (Combur¹⁰ Test, Boehringer Mannheim) proteinuria dipsticks. Negative samples were further tested with Micral Test strips (Boehringer Mannheim, BM) and Microalb Turbidimetry Tests (Ames)

PRODUCTION OF PRIMARY ANTISERUM

Rabbits were immunised by intra-muscular injection with a primary dose of 100 μg pure human albumin in Freund's complete adjuvant, followed by multi-site subcutaneous 100 μg booster doses at monthly intervals. The first test bleed was obtained after the third booster. Antiserum was frozen and stored at -20°C or

lyophilised and stored at 4°C. This primary antiserum rabbit anti-human albumin (RaH-Alb) was used without any further purification and diluted accordingly in assay buffer. The titre and specificity of the antiserum was characterised and determined by ELISA.

PREPARATION OF HRP-LABELLED ALBUMIN

Human albumin was conjugated to enzyme horse radish peroxidase, HRP (Sigma) by the metaperiodate method.

ELISA PROCEDURES

Antigen coated ELISA

Human albumin was adsorbed onto wells of microtitre ELISA plates by incubating in each well 1 $\mu\text{g}/100 \mu\text{l}$ in coating buffer at 4°C overnight. Unbound sites were blocked with 200 μl PBS gelatin blocking solution for 1 h at 37°C. All subsequent steps were performed at room temperature. After washing three times with wash solution PBS.Tween and drying, 50 μl albumin standard solutions (10 - 625 $\mu\text{g}/\text{l}$), quality control samples or urine samples (prediluted 1:20 or 1:100) were added to duplicate wells. After a further addition of 50 μl primary antiserum RaH-Alb diluted 1:60,000 in assay buffer, the plates were placed on a plate-shaker and left to incubate for 2 hours. The mixtures were discarded and the plates were washed three times and dried. Secondary antiserum goat anti-rabbit immunoglobulin labelled with horse radish peroxidase (GaR-IgG-HRP) (Bio-Rad) diluted 1:10,000 in assay buffer was then added to each well (100 μl). After incubation of 2 hours, the plates were again washed three times and 100 μl substrate solution was added. The plates were incubated in the dark for the enzyme reaction to proceed. The chromogenic reaction was terminated after 30 minutes by addition of 100 μl stopping solution. An ELISA plate reader (AT 400 SLT, SLT Instruments, Austria) was used to measure optical density using the dual wavelength mode at 492 nm and reference at 620 nm. Data analysis and curve fitting was performed using the LKB-Wallac RIA-Calc programme.

Antibody coated ELISA

Rabbit anti-human albumin serum diluted 1:20,000 in coating buffer was adsorbed onto wells of microtitre ELISA plates (100 μl per well). After blocking and washing, 50 μl albumin standards or prediluted urine samples were incubated for 1 hr in duplicate wells together

with 50 ul albumin-HRP (0.5 ng). The plates were washed and then incubated in the dark for 30 mins with 100 ul substrate solution for colour development.

CREATININE ANALYSIS

For measurement of creatinine concentrations in urine samples, the Jaffe method was used. A micro-method was established that used 150 ul urine samples diluted 1:100 fold, 50 ul picric acid and 50 ul 0.75 M NaOH incubated in wells of ELISA plates. The standard curve range was 40 - 200 $\mu\text{mol/l}$.

DATA ANALYSIS

Normality of data distribution was examined by statistical tests for chi-square goodness of fit. Student's unpaired t test was used to compare group means, with reference ranges reported as mean \pm 2SD. Non-parametric methods were used for data not normally distributed. Correlation analyses were performed using linear regression. Values of $p < 0.05$ were considered significant.

RESULTS

Antiserum specificity: The RaH-Alb antiserum was found to be specific for albumin as the cross-reactivities against haemoglobin was 0.09% and 0.01% against immunoglobulin G (IgG).

ELISA optimisation: The optimum amount of albumin for coating onto ELISA wells was determined by measuring the optical densities for total binding (B_o) and non-specific binding (NSB) and full standard curves with various albumin concentrations 0.25, 0.5, 1.0, and 2.0 ng/100 ul. The optimum albumin concentration for coating was found to be 1.0 ng/100ul.

The optimum working titres for the primary antiserum RaH-Alb and secondary antiserum GaR-IgG-HRP were tested using dilutions of 1:20,000, 1:40,000, 1:60,000 and 1:80,000 and 1:5,000, 1:10,000, 1:20,000 respectively. The appropriate titres that gave $B_o > 0.6$ and NSB < 0.02 OD units were 1:60,000 for RaH-Alb and 1:10,000 for GaR-IgG-HRP. The incubation times were fixed at 2 hours as shorter incubation times reduced B_o values.

For the antibody coated ELISA, an optimum RaH-Alb antibody dilution of 1:20,000 and binding of 0.5 ng albumin-HRP, 1 hr incubation time were obtained.

ELISA validation: The standard curve ranged from 10 - 625 $\mu\text{g/l}$ and the sensitivity of the assay was 16 - 28 $\mu\text{g/l}$. Recovery of albumin concentrations at 20, 80 and 320 $\mu\text{g/l}$ were 109.2 \pm 11.9%, 102.6 \pm 6.2% and 100.6 \pm 3.9% respectively. Dilution of medium and high QC samples 1:2 to 1:16 fold gave linearity recovery results of 91.3 \pm 1.7% and 94.4 \pm 7.6% respectively.

The precision of the in-house ELISAs were found to range 3.9% to 10.2% for the intra-assay coefficients of variation (CVs) for the concentration range of 20 - 320 $\mu\text{g/l}$. The corresponding inter-assay CVs were 2.6% to 11.9%.

Comparison studies: A total of 152 urine samples with microalbumin concentrations range 10 - 300 mg/l were simultaneously assayed using the two in-house ELISAs. A good correlation of $r = 0.996$ ($y = 0.93x + 8.0$, $p < 0.001$) was obtained (Table 1). When compared to commercial kits, the in-house ELISA gave $r = 0.961$ ($y = 1.03x + 5.2$, $p < 0.001$, $n = 115$) versus the Micral Test strips (Boehringer Mannheim) and $r = 0.94$ ($y = 0.93x + 9.8$, $p < 0.001$, $n = 80$) versus the turbidimetry kit (Urin Pak Immuno Microalb, Ames).

TABLE 1: Comparison of in-house antigen coated ELISA

Assays	Sample size n	Correlation: coefficient r	equation $y = mx + c$	probability P
In-house Antibody coated ELISA	n=152	$r = 0.996$	$y = 0.93x + 8.0$	$p < 0.001$
Microalb (Ames) Turbidimetry Tests	n=80	$r = 0.940$	$y = 0.93x + 9.8$	$p < 0.001$
Micral (BM) Test Strips	n=115	$r = 0.961$	$y = 1.03x + 5.2$	$p < 0.001$

TABLE 2: Microalbumin excretion in normal population as measured by ELISA

Urine sample	n	Mean	SD	Range	
12-h urine	40	2.5	1.4	0.7 - 5.3	mg/12h
AER	40	3.5	1.9	1.0 - 7.3	ug/min
FMU	40	2.7(median)		0.1 - 10.2	mg/l
FMU _a /FMU _c	40	0.4	0.2	0.1 - 0.9	mg/mmol
RU	40	8.3(median)		0.8 - 26.1	mg/l
RU _a /RU _c	40	0.8	0.4	0.2 - 1.6	mg/mmol

It was also shown that ELISA can also be used to measure macroalbuminuria (>300 mg/l) after appropriate further dilution before assay. When compared to results from Albustik proteinuria dipstick tests that gave semi-quantitative values of 0.3, 1.0 and 5.0 g/l, ELISA values were 0.3 ± 0.05 (n=10), 1.0 ± 0.25 (n=6) and 4.0 ± 1.0 (n=13) g/l respectively.

Normal reference ranges for microalbuminuria: Table 2 shows the microalbumin concentrations in normal urine samples collected 12h overnight or spot urine samples collected early in the morning or randomly. Albumin excretion rate (AER) was calculated from 12h samples, while microalbuminuria values from spot urine samples were divided by individual creatinine concentrations. The normal microalbumin reference ranges (mean \pm 2SD) for 12h samples and AER were 0.7 - 5.3 mg/12h and 1.0 - 7.3 ug/min respectively.

For samples from first morning urine (FMU) and random urine (RU) the 95% confidence limits were 0.1 - 10.2 mg/l (median 2.7) and 0.8 - 26.1 mg/l (median 8.3). Data from microalbuminuria values corrected for creatinine excretion were normally distributed and their reference ranges were 0.1 - 0.9 mg/mmol for FMU_a/FMU_c and 0.2 - 1.6 mg/mmol for RU_a/RU_c. Both microalbuminuria values from RU samples were significantly higher (p<0.001) than values from FMU samples. Results from linear regression analysis (Table 3) showed that FMU_a/FMU_c and RU_a/RU_c values from spot urine samples correlated with each other (r=0.51, p<0.01) and with AER (r=0.52 and r=0.56, p<0.001).

The intra-individual CVs (n=6) for AER, FMU and FMU_a/FMU_c were $44.6 \pm 8.8\%$, $41.4 \pm 28.8\%$, $30.4 \pm 14.2\%$ respectively.

TABLE 3: Correlation of microalbumin excretion in various urine samples

Urine sample	n	r	y=mx+c	P
FMU vs AER	40	0.47	y=0.82x+0.83	<0.01
FMU _a /FMU _c vs AER	40	0.52	y=0.06x+0.07	<0.001
RU vs AER	35	0.51	y=1.1x +4.73	<0.01
RU _a /RU _c vs AER	35	0.56	y=0.05x+0.56	<0.001
RU _a /RU _c vs FMU _a /FMU _c	35	0.51	y=0.13x+0.21	<0.01

TABLE 4: Microalbuminuria testing by ELISA in diabetic patients

Urine sample	Total	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
RU		<26.1	26.1-300	>300 mg/l
	338	162 (47.1%)	133 (39.3%)	43 (12.7%)
RU _a /RU _c		< 1.6	1.6-25	>25 mg/mmol
	338	115 (34.0%)	162 (47.9%)	61 (18.0%)

Microalbuminuria analysis in diabetic patients: Abnormal albumin excretions in diabetic patients were tested using spot random urine samples and ELISA. Table 4 shows that 52.1% of 338 diabetic patients showed abnormal raised albumin excretion based on RU values >26.1 mg/l while the percentage was higher at 66.0% based on RU_a/RU_c values >1.6 mg/mmol. Based on RU values, 39.3% had microalbuminuria (26.1 - 300 mg/l) while 12.7% had macroalbuminuria (>300 mg/l). The corresponding percentages using RU_a/RU_c values corrected for creatinine concentrations were 47.9% and 18.0% respectively.

DISCUSSION

Two in-house ELISAs for microalbuminuria measurement were established and validated. For analytical recovery and linearity measurements, good results within $100 \pm 10\%$ were obtained. The in-house ELISA assay standard curve range was 10 - 625 μ g/l and the assay sensitivity of 0.4 mg/l for urine samples diluted 1/20 fold was obtained. This sensitivity is comparable to RIA methods (0.1-0.5 mg/l) but is twenty fold greater than the minimal detectable concentrations of 3.0-5.0 mg/l for immunoturbidimetry and immunonephelometry.^{6, 7, 12} The precision (intra-CVs $<10\%$ and inter-CVs $<12\%$) of the ELISAs are also comparable to RIA and other methods with CVs ranges of 6.5 - 9.0%. The antibody coated ELISA is simpler and shorter (total time of 2 hours) compared to antigen coated ELISA which will take 5 hours to complete. When compared to two commercial microalbuminuria kits, excellent correlations of $r > 0.90$ were obtained. The advantages of the in-house ELISAs as compared to commercial assays include low cost and availability.

Microalbuminuria analysis in normal population was performed using urine samples from three different types of collection, 12-h overnight, first morning and random. Our upper limits of normal for AER was 7.3 μ g/min, 5.3 mg/l for FMU and 0.9 mg/mmol for FMU_a/FMU_c which are in agreement with those of earlier reports.^{8, 16-18} It was further proposed that AER values of 7.6 - 30 μ g/min represent borderline microalbuminuria and should therefore be considered as abnormal.⁸ Although AER measurements are required for confirmation of microalbuminuria, the use of untimed urine specimens for screening purposes have been proposed. Both first morning and random urine

samples have been used. However, it is still not certain whether there is a need to consider the variations in urine volume and to express microalbuminuria values as albumin:creatinine ratios for untimed samples.²⁰⁻²⁴ We have confirmed the earlier findings^{19, 20} shown in this study that both first morning (FMU) and random (RU) urine specimens gave microalbuminuria results that correlated ($p<0.01$) well with AER results. The correlations were even better ($p<0.001$) for results corrected for creatinine and there were less intra-individual variations for FMU samples. Furthermore, FMU_a/FMU_c were significantly lower, showed less variations and within a closer range (0.1 - 0.9 mg/mmol) when compared to results from random samples (0.2 - 1.6 mg/mmol). We have further confirmed the previously reported¹⁸ cut-off limit of 1 mg/mmol for FMU_a/FMU_c . Thus, first morning urine samples can be used as a screening test for detection of microalbuminuria, to be confirmed by AER measurements.

We used the ELISA to test for microalbuminuria on random urine samples from diabetic patients. A high percentage of 52.1% patients showed abnormal albumin excretion based on RU values >26.1 mg/l, while a higher percentage of 66.0% was obtained based on RU_a/RU_c values >1.6 mg/mmol. Amongst those patients that showed abnormal albumin excretion, at least 12.7% or 18.1% had markedly elevated albumin excretion in the proteinuria or macroalbuminuria range (> 300 mg/l or > 25 mg/mmol). We believe that the false negative results based on RU values are probably due to dilution effects of polyuria in poorly controlled diabetic patients. Although these preliminary results need to be further confirmed by repeated testing and AER measurements, we recommend early identification of diabetic nephropathy in diabetic patients should be performed through microalbuminuria measurements using sensitive tests such as our in-house ELISAs.

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