



Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

On-site determination of microalbuminuria based on Particle-Enhanced Turbidimetric-Inhibition Immunoassay (PETINIA) by portable fiber-optic spectrometer

Warattaya Sinwat¹, Assawapong Sappat², Adisorn Tuantranont² and Wanida Laiwattanapaisal^{3*}

¹Graduate Program in Clinical Biochemistry and Molecular Medicine, Faculty of Allied Health Sciences, Chulalongkorn University, Patumwan, Bangkok, 10330, Thailand

²Nanoelectronics and MEMS Laboratory, National Electronics and Computer Technology Center, Pathum Thani, 12120, Thailand

³Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Patumwan, Bangkok, 10330, Thailand

ABSTRACT

A homemade fiber-optic spectrometer has been fabricated for portable and on-site determination of microalbuminuria. The principle of the method was based on particle-enhanced turbidimetric-inhibition immunoassay (PETINIA), which was not affected by the hook effect phenomenon. This method was linear from 0 to 150 mg L⁻¹ ($r^2 = 0.987$), with a detection limit of 3.01 mg L⁻¹ (S/N = 3). The intra-assay coefficient of variations (CVs) ranged from 3.1 - 4.8%, and the inter-assay CV was 6.7%, as assessed by calibration curves performed on three consecutive days. The precision of 10 replicate measurements of 50 mg L⁻¹ HSA in a single analytical run was 6.5%. Interfering compound tests were satisfactory with recoveries of 95.0% to 105.8%. The system demonstrated its applicability to perform accurate measurements outside the laboratory for in-field analysis of the microalbuminuria (MAU) assay. The albumin concentrations in real urine samples measured by this system correlated well with the particle-enhanced turbidimetric immunoassay performed on a conventional spectrophotometer with $r^2 = 0.996$ (n = 58).

Keywords: Microalbuminuria, particle-enhanced turbidimetric-inhibition immunoassay (PETINIA), hook effect phenomenon, on-site determination, diabetic nephropathy

INTRODUCTION

The detection of low protein content in urine is very important for clinical diagnosis, especially in the stages of microalbuminuria (MAU). MAU is defined as the presence of minute amounts of albumin in urine (30 - 300 mg/day) and is one of the signs of early glomerular dysfunction often seen in diabetic nephropathy [1-3]. The importance of annual testing for MAU in every diabetes patient was perceived by The American Diabetic Association, who have issued this guidance since 2007 [4]. The measurement of MAU has been reported to be a useful marker for monitoring hypertension and cardiovascular disease in nondiabetic patients [5-9]. Many analytical methods have been proposed for determining MAU. These analytical methods can be classified as qualitative and quantitative measurement. Lateral flow immunochromatography was generally used for screening as a qualitative or

semi-quantitative assay [8, 10, 11]. However, to confirm the stage of MAU, quantitative immunoassays such as enzyme linked immunosorbent assay (ELISA) [12, 13], immunonephelometry [14, 15], immunoturbidimetry [16, 17], radioimmunoassay [18, 19] and particle-enhanced immunoturbidimetric immunoassay (PETIA) [20, 21] have been utilized. Among these quantitative immunoassays, the methods based on immunonephelometry, immunoturbidimetry and PETIA have been recognized and widely used for determination of MAU in most clinical laboratories [22, 23]. These methods are homogeneous immunoassays so they are able to be performed on most of the automated chemistry analyzers that are most often available in general laboratories. Although these methods provide good sensitivity and reproducibility, they could give a false negative result caused by antigen excess, known as the high dose hook effect [24-27].

Urine albumin, markedly increased in patients with uncontrolled diabetes, may lead to falsely low results when assayed with conventional immunoturbidimetric assays. To avoid these false negative results, additional testing to detect antigen excess must include, e.g., qualitative measurement of urinary albumin by urine dipstick [28, 29], quantitative measurement of urinary total protein [26], or multiple dilutions of the sample before albumin assay [30]. However, when the assay is performed with an automated analyzer, most of the commercial assay kits provide leaflet information about the zone effect with the protocol of “prozone check” for every sample. The prozone check involves adding additional albumin after the measurement is completed and then observing the change in absorbance. All of these procedures are complicated and costly and, hence, affect the overall assay cost of MAU. For in-field analysis using a simple, low-cost method, the step of the prozone check is obviously not required.

Methods based on particle-enhanced turbidimetric inhibition immunoassay (PETINIA) were successful in retaining the sensitivity of the method using latex beads without affecting the prozone phenomenon. The principle of PETINIA has been recognized for a decade and these principles have been applied in several clinical analyses such as albumin in urine [31], theophylline therapeutic drugs [32], and serum carbamazepine [33]. The PETINIA method is based on the inhibition of the particle agglutination reaction by the competitive binding of polyclonal antibody to the antigen-immobilized beads and the antigen in the sample. The rate of absorbance increase is inversely proportional to the concentration of analytes in the sample.

In this paper, PETINIA was utilized to determine microalbuminuria from real urine samples. To demonstrate its applicability for in-field analysis, the portable fiber-optic system was set up to analyze MAU from diabetes patients at a sub-district health promotion hospital (Ratchaburi, Thailand), and the results were compared with the conventional particle-enhanced turbidimetric immunoassay.

EXPERIMENTAL SECTION

2.1 Apparatus

A UV-VIS Spectrophotometer (Evolution 600, Thermo Scientific, USA) using quartz cuvettes with an optical path length of 1 cm was used. The temperature of the experiment was set at 37 °C by means of a Peltier temperature-controlled system. The absorption data were recorded using Visual Lite operating software provided by the manufacturer.

The portable system components for the light adsorption immunoassay are shown in Fig.1. USB 4000 spectrometer (Ocean Optic), tungsten light source (LLS), and fiber-optic cables were assembled and arranged in a plastic box, with dimensions of 25 x 22 x 9 cm. The programmable temperature controller, used to maintain the optimal temperature during the assay, operated with a cartridge heater and a temperature sensor (thermocouple). The light emitted by the tungsten light source passed through a cuvette containing sample solutions and illuminated the photodetector. Absorbance and spectrum changes, recorded using a uniquely developed program derived from SpectraSuite (Ocean Optics) operating software provided by manufacturer of the spectrometer, runs on a portable computer.

2.2 Chemicals and immunoreagents

Highly carboxylated polystyrene/polyacrylic acid (10% solids- diameter 0.51 μm) latex microspheres were purchased from Bangs Laboratories, Inc., Fishers, IN. Human serum albumin (HSA), rabbit anti-human albumin antibody, bovine serum albumin (BSA) and Tween-20 were obtained from Sigma-Aldrich (St. Louis, MO). MES monohydrate was purchased from USB Corporation (Cleveland, USA). Sodium chloride (NaCl), potassium dihydrogen phosphate (KH₂PO₄) and dipotassium hydrogen phosphate (K₂HPO₄) were purchased from Merck

(Darmstadt, Germany). Polyethylene glycol 6000 (PEG 6000) was purchased from Fluka (Buchs, Switzerland). Hemoglobin, gamma globulin, ascorbic acid, D-(+)-glucose, urea, uric acid and creatinine used in the interference studies were from Sigma (St. Louis, MO). Ampicillin and bilirubin were from Fluka (Buchs, Switzerland).

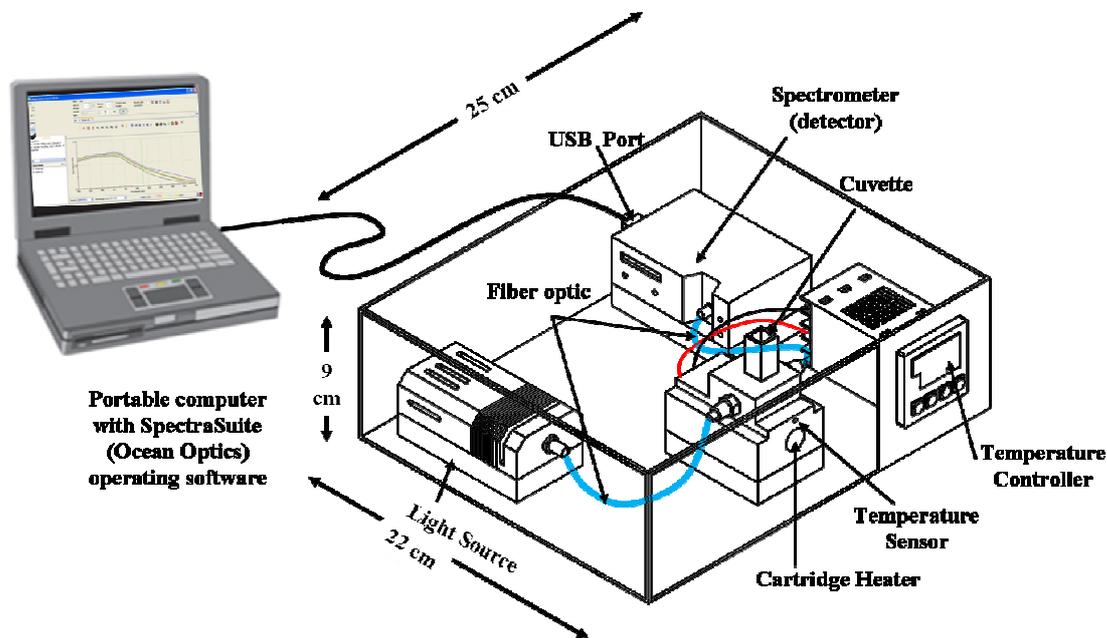


Fig. 1. The portable system set up for the light adsorption immunoassay.

2.3 Buffers and standards

All solutions were prepared in Milli-Q water, except the albumin standards that were prepared in 0.9% NaCl solution. The adsorption buffer and washing buffer was 25 mM MES buffer, pH 4.5. The blocking and storage buffer was 10 mM sodium phosphate buffer, pH 7.4 containing 1% BSA and 0.05% Tween-20.

Unless otherwise stated, an assay buffer was 20 mM potassium phosphate buffer (pH 7.4) containing 200 mM NaCl and 6% PEG. The buffer solutions were filtered with 0.45-micron membrane filters (Pall Corporation, USA) and kept at 4 °C until use.

2.4 Sample Preparation

Ethical approval for the study was obtained from The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University. Spot urine samples were collected from diabetes patients at sub-district health promotion hospitals in Banpong, Ratchaburi, Thailand. The urine samples were centrifuged at 1,500 rpm for 5 minutes prior to subjecting the clear supernatant to the assay.

2.5 Bead immobilization

HSA were immobilized onto the highly carboxylated latex beads by a passive adsorption protocol. For each batch of immobilization reactions, 40 μ L of 10% latex beads were diluted in 360 μ L of washing buffer utilizing 4 mg of beads. Beads were washed twice with washing buffer by centrifugation at 9,300 rpm for 10 minutes. After the supernatant was discarded, 400 μ L of HSA (137 μ g/mL) dissolved in the adsorption buffer was added to the bead pellets. The suspension was gently mixed overnight at 4 °C before centrifugation to remove the supernatant. To block the non-immobilized beads, blocking buffer was added and the beads were resuspended for an additional 30 minutes. The supernatant was subsequently removed by centrifugation, and the immobilized beads were resuspended in 400 μ L of storage buffer for use as the stock immobilized beads solution and were maintained at 4 °C until used. For each batch of immobilizations, 400 μ L of stock immobilized bead solution can be used for 80 reactions.

2.6 Immunoassay procedures

Before measurement, the tungsten light source of the portable system was turned on to pre-warm for at least 15 minutes. To prepare the working bead solution, the stock immobilized beads were diluted 10 times with 10 mM sodium phosphate buffer, pH 7.4. The optical density (OD) of the working immobilized bead solution at 510 nm was approximately 0.5-0.6, with the assay buffer set to zero. The mixture of 12 μL standard or urine sample and 13 μg of the albumin antibody were gently mixed in 388 μL of an assay buffer and allowed to form the antigen-antibody complex for 2 minutes at 37 $^{\circ}\text{C}$. At this time, 50 μL of the working immobilized beads was added to the pre-warmed mixture of antibody-antigen complexes and immediately subjected to absorbance measurement at 510 nm. An increase in absorbance was monitored for 2 minutes at 37 $^{\circ}\text{C}$. For each experiment, the two levels of commercial-quality control urine were assayed along with samples and standards. Each urine sample was measured in duplicate.

RESULTS AND DISCUSSION

3.1 Effect of NaCl on the immunoreaction

NaCl was added to all assay buffers to enhance the immunoturbidimetry reaction [34]. Because most of the immunoreactions were performed under physiological conditions at pH 7.4 with an ionic strength between 150-170 mM [34, 35], NaCl concentrations up to 400 mM were investigated to determine the optimal conditions in our system. While the NaCl concentrations were varied, the PEG concentration was fixed at 6 %.

The results show that increasing the NaCl concentration increased the rate of reaction (Fig. 2). However, concentrations of NaCl higher than 300 mM can lead to a sharp decline in the assay signals. At 200 mM NaCl, the difference in the assay signal between 25 and 100 mg L^{-1} HSA was slightly larger than the difference at 300 mM NaCl. The concentration of 200 mM NaCl was therefore selected and used for subsequent experiments because this concentration consumed less chemicals.

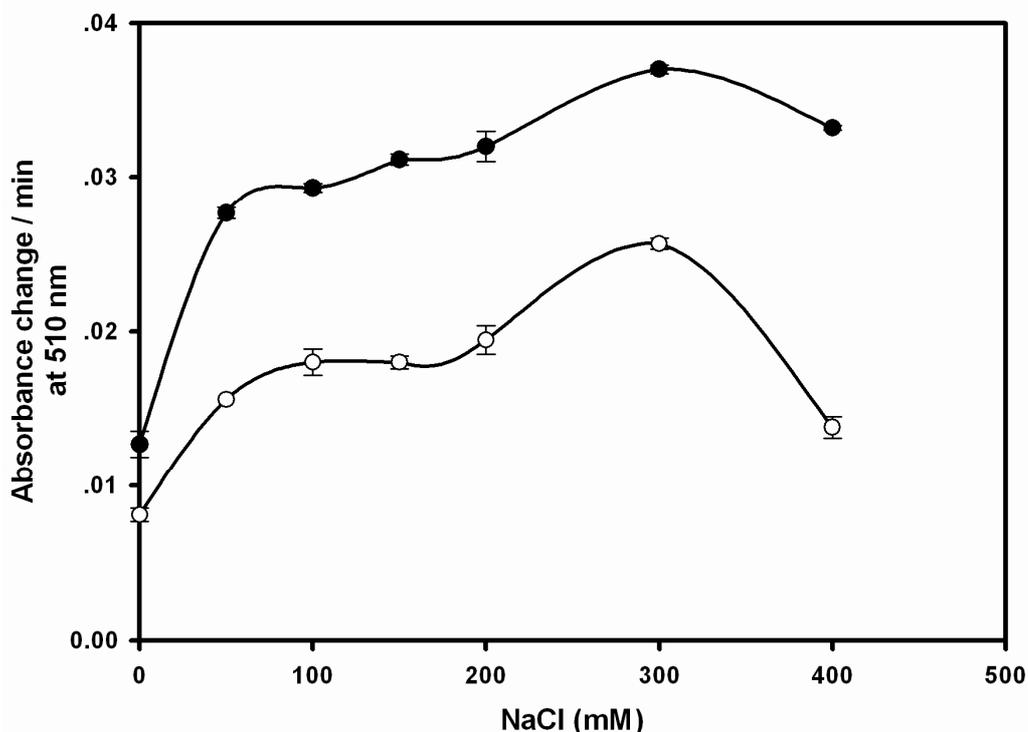


Fig. 2. Effect of varying NaCl concentrations on the assay signal based on PETINIA.
Solid circles (●): 25 mg L^{-1} and open circles (○): 100 mg L^{-1} of HSA standard

3.2 Effect of PEG on the immunoreaction

PEG present in the buffer has been reported to help to accelerate the immunoreaction [36]. The concentration of PEG affects the immunoagglutination by maintaining the colloidal stability [37, 38]. In addition, the optimized amount of PEG helps to increase the sensitivity of the assay, thereby influencing the range of the assay [39]. In our study, PEG values ranging from 0-12% were investigated to determine the optimal concentration. As the results in Fig. 3 show, the rate of the immunoreactions was increased with increasing PEG concentration up to 8%. However, PEG concentrations higher than 8% did not significantly improve the assay signals. At 6% PEG, the difference in the assay signals between 0 and 75 mg L⁻¹ HSA was clearly observed and approximately twice as large as the signals obtained using 8% PEG. Using the buffer combined with 6% PEG produced the steepest slope when generating an analytical curve for the MAU assay with good sensitivity of the assay.

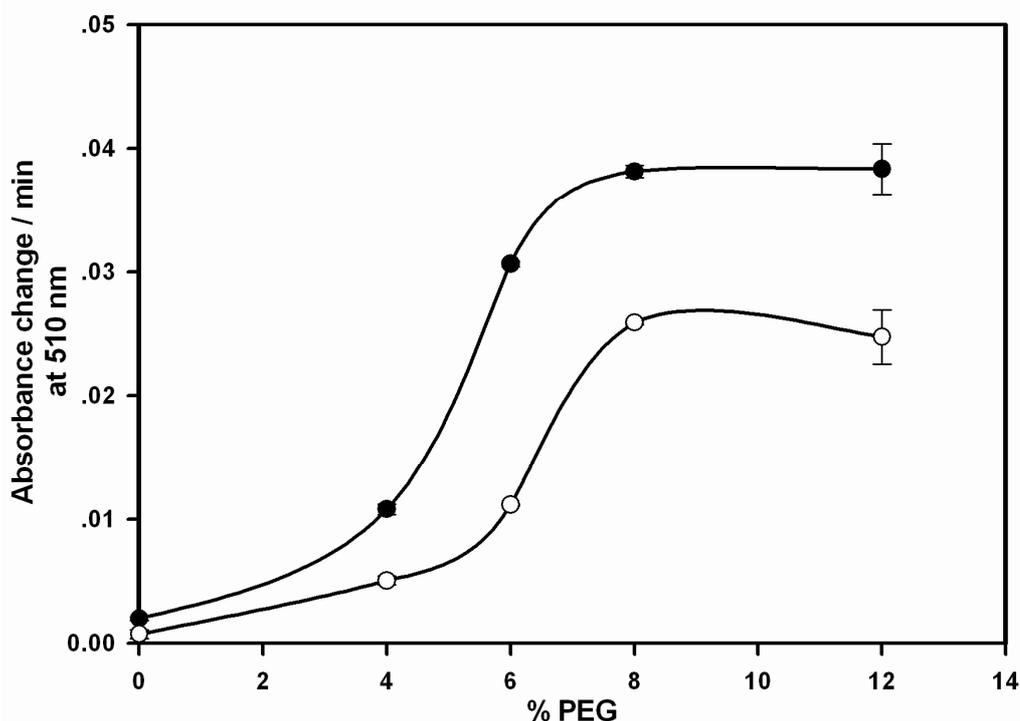


Fig. 3. Effect of PEG concentration on the assay signal based on PETINIA. Solid circles (●): 25 mg L⁻¹ and open circles (○): 100 mg L⁻¹ of HSA standard

Table 1 Effects of substances that interfere with urine albumin by PETINIA

Added substance	Interferences added (mg L ⁻¹)	% Recovery
None	0	100.0%
Urea	5000	99.1
Creatinine	1000	102.6
Uric acid	500	105.8
NaCl	1000	100.5
KCl	5000	105.8
Ascorbic acid	150	100.3
Glucose	10000	100.0
Gamma globulin	1000	95.0
Hemoglobin	50	100.8
Bilirubin	6.25	102.0
Ampicillin	10	97.1

3.3 Interference from other substances

We evaluated the effect of the various substances and drugs that could possibly interfere with the proposed method. The results shown in Table 1 indicate that 500 mg L⁻¹ of uric acid and 5000 mg L⁻¹ KCl might slightly disturb our

method, but a recovery of 105.8% was obtained. These minute interferences could be explained by a cross-reaction of the antibody with proteins other than albumin, especially at extremely high concentrations. However, some compounds such as urea, creatinine, gamma globulin, hemoglobin, ampicillin, ascorbic acid, and glucose were not found to interfere significantly with our method. Recoveries between 95% and 102.6% were obtained in the presence of the compounds listed.

3.4 Precision and reproducibility

HSA standards with different concentrations were prepared and stored at $-20\text{ }^{\circ}\text{C}$ to study the precision and reproducibility of this method. The intra- and inter-assay precision was calculated from three calibration curves performed on three consecutive days. The results demonstrated that intra-assay coefficient of variations (CVs) ranged from 3.1 - 4.8%, and the inter-assay CV was 6.7%. The precision of 10 replicate measurements of one aliquot of 50 mg L^{-1} HSA in a single analytical run was determined to be 6.5%.

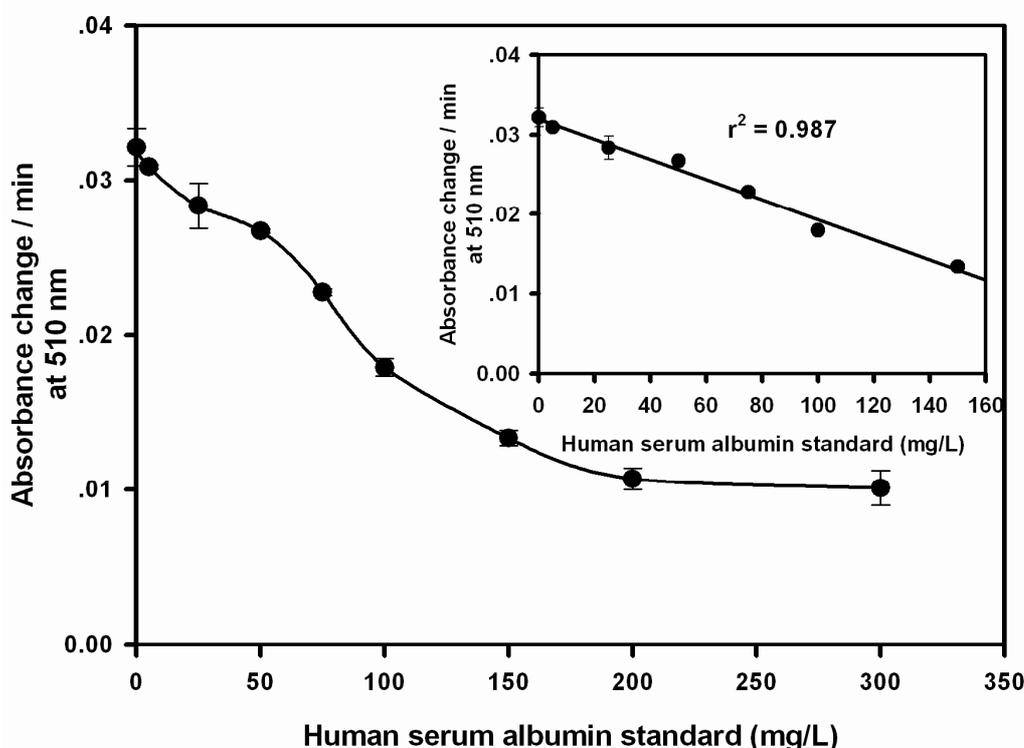


Fig. 4. Analytical plot for the determination of microalbuminuria based on PETINIA performed with portable fiber-optic spectrometer.

3.5 Analytical curve

A 10 mg mL^{-1} stock solution of HSA in 0.9% NaCl was serially diluted to give concentrations of 0, 5, 25, 50, 75, 100, 150, 200 and 300 mg L^{-1} . All solutions were analyzed and their rates of reactions were constructed for a calibration curve. The calibration curve is shown in Fig. 4. In this study, the linear range for detection of albumin was 0 to 150 mg L^{-1} ($r^2 = 0.987$). The limit of detection (LOD) was calculated from ten replicate assays of the blank sample. Based on a signal-to-noise ratio (S/N) of 3, the detection limit determined for albumin was 3.01 mg L^{-1} . Our proposed method was slightly less sensitive than the previously described method based on immunoturbidimetry, where the detection limit was 2 mg L^{-1} [38]. When compared to other methods based on PETINIA for albumin, the sensitivity and linear range of our method were lower than the previous report that utilized the Dade Aca[®] analyzer [31], where the LOD and linear range were 0.4 mg L^{-1} and $2 - 250\text{ mg L}^{-1}$, respectively. The broader linear range of this method was obtained because it consumed the antibody considerably more than our method, where $20\text{ }\mu\text{g}$ is required per assay. The automated analyzer is generally large and limited to use only in laboratories. The sensitivity

and linear range produced by the portable fiber-optic spectrometer were sufficient to determine MAU, and the portability of this unit is beneficial for on-site analysis.

(A)

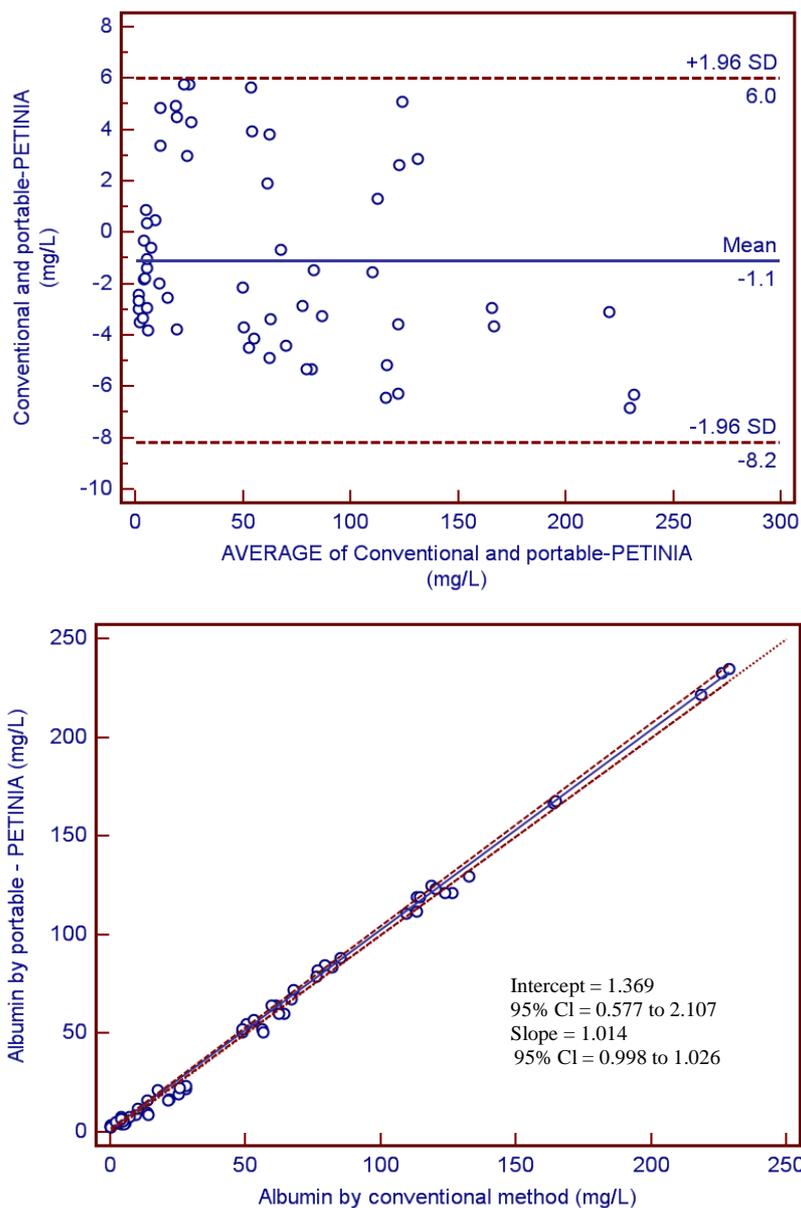


Fig. 5. Comparison of results from the portable fiber-optic spectrometer system and the conventional PETINIA for determining MAU. Results shown with (a) Bland-Altman bias plot and (b) Passing-Bablok regression analysis.

3.6 Method comparison

The need for the rapid, easy and on-site diagnosis has motivated us to develop a portable microalbuminuria system for clinical diagnostics, especially in urban healthcare services for most developing countries.

For comparative studies, 58 urine samples from diabetes patients at a sub-district Health Promotion Hospital were analyzed for MAU by our proposed method. The results were compared to the results from the conventional particle-enhanced turbidimetric immunoassay kit obtained from Biosystems S.A. (Barcelona, Spain) with the analysis performed by a conventional spectrophotometer. To obtain quantitative measurement of MAU higher than

the assay limit ($>150 \text{ mg L}^{-1}$), the samples were necessarily diluted with 0.9% NaCl solution and re-assayed. The results obtained from both methods were analyzed with the Bland-Altman test [40], and these results are shown in Fig. 5A. The results demonstrated that there was no statistically significant difference between the results obtained by two methods. The average bias for the conventional method versus PETINIA was within the mean value ± 1.96 SD. The agreement between PETINIA and the conventional particle-enhanced turbidimetric immunoassay was also assessed by the Passing-Bablok regression [7], as shown in Fig 5B. According to our study, the regression equation given by the conventional method versus PETINIA was $y = 1.014x + 1.369$, where the slope was not significantly different from 1 and the intercept was not significantly different from 0. These results indicated that, for the determination of microalbuminuria, the portable fiber-optic spectrometer was in good agreement with the conventional particle-enhanced turbidimetric immunoassay.

3.7 Detection of prozone effect

To investigate the proposed method's ability to detect antigen excess, 5 urine samples showing high albumin content were analyzed as non-diluted and diluted samples. The results are shown in Table 2. These results demonstrate that the albumin values higher than 1000 mg L^{-1} were determined to be falsely lowered below their actual value when assayed with the immunoturbidimetric assays using non-diluted samples. Without the prozone check, those samples would lead to misdiagnosis because their actual albumin concentrations were relatively high and could be classified as proteinuria. The prozone check is necessary for every sample when the assay is performed with conventional immunoturbidimetry. This approach is laborious and increases the assay cost. Samples with extremely high albumin concentrations were not frequently found. If those samples were subjected to analysis by our PETINIA methodology, only the extremely high samples would be reported as containing albumin levels greater than 150 mg L^{-1} and would be suggested for a further dilution step to determine the actual albumin content. For dilutions of 100- to 1000-fold in the samples before re-analysis, results from both methods were similar.

Table 2 Detection of the prozone effect in two methods

Sample	Undiluted samples (mg L^{-1})		Diluted samples (mg L^{-1})	
	Immunoturbidimetric assays	PETINIA	Immunoturbidimetric assays	PETINIA
1	2.6	198.9 *	29674.7	29006.8
2	14.9	191.6 *	5620.0	5991.0
3	30.0	184.1 *	2373.9	2570.4
4	83.8	183.0 *	1280.7	1244.5
5		91.0 167.5 *	1036.2	1133.2

* The values were above the upper assay range of the proposed PETINIA ($> 150 \text{ mg L}^{-1}$). These results suggested a sample dilution and re-assay.

CONCLUSION

PETINIA was proposed as an effective method for determining low levels of urinary albumin and demonstrated its feasibility for accurate and sensitive determination of MAU using a portable spectrometer. The linear range of $0\text{-}150 \text{ mg L}^{-1}$ albumin covers the entire range of normal and MAU values, indicating that this method is desirable for the clinical laboratory and for helping physicians to diagnose early diabetic nephropathy.

Patients with MAU higher than 150 mg L^{-1} will not be misdiagnosed as a result of the hook effect. A re-check for the hook effect phenomenon was not necessary with our portable device. The portable fiber-optic spectrometer was successfully used and proved to be very feasible for use outside the laboratory because of its small size and simple and rapid operation. The system demonstrated its applicability for sensitive and accurate assay of MAU by using the in-house preparation of the beads. In conclusion, this proposed method is very beneficial for on-site analysis of MAU in diabetes patients, in the rural and remote areas, especially of developing countries.

Acknowledgments

Authors acknowledge the Cluster and Program Management Office, National Science and Technology Development Agency (NSTDA) for financial support.

REFERENCES

- [1] CE Mogensen. *Kidney Int.*, **1987**, 31, 673-689.

- [2] JL Gross; MJ de Azevedo; SP Silveiro; LH Canani; ML Caramori; T. Zelmanovitz. *Diabetes Care*, **2005**, 28(1), 164-76.
- [3] Rambhade S; Chakraborty A. *J. Chem. Pharm Res.*, 2010, 2(6), 7-25.
- [4] I. PREVENTION, *Diabetes care*, **2007**, 30, 1.
- [5] R Bigazzi; S Bianchi; D Baldari; VM Campese. *J.Hypertens.*, **1998**, 16(9), 1325-33.
- [6] K Klausen; K Borch-Johnsen; B Feldt-Rasmussen; G Jensen; P Clausen; H Scharling; M Appleyard; JS Jensen. *Circulation*, **2004**, 110(1), 32-5.
- [7] H Passing; W Bablok. *Clin. Chem.Lab. Med.*, **1983**, 21(11), 709-720.
- [8] JP Garg; GL Bakris. *Vasc. Med.*, **2002**, 7(1), 35-43.
- [9] Sreenivasan R S; Vanitha C; Ezhamani G. *J. Chem. Pharm Res.*, 2011, 3(4), 92-102
- [10] B Agrawal; A Berger; K Wolf; FC Luft. *J.Hypertens.*, **1996**, 14(2), 223-8.
- [11] S Leong; K Lui; W Ng; A Thai. *Singapore Med. J.*, **1998**, 39(3), 101-3.
- [12] TL Wu; PY Chang; CC Li; KC Tsao; CF Sun; JT Wu. *Ann. Clin. Lab. Sci.*, **2005**, 35(2), 149-54.
- [13] C Aybay; R Karakus. *Turk. J. Med. Sci.*, **2003**, 33(1), 1-6.
- [14] R Stamp. *Ann.Clin.Biochem.*, **1988**, 25, 442-3.
- [15] CD Deaton; KW Maxwell; RS Smith; RL Creveling. *Clin. Chem.*, **1976**, 22(9), 1465.
- [16] L Paloheimo; M Pajari-Backas; E Pitkanen; L Melamies; R Rissanen. *Clin. Chem.Lab. Med.*, **1987**, 25, 889-92.
- [17] O Giampietro; G Penno; A Clerico; L Cruschelli; A Lucchetti; M Nannipieri; M Cecere; L Rizzo; R Navalesi. *Acta Diabetol.*, **1992**, 28, 239-45.
- [18] J Woo; M Floyd; DC Cannon; B Kahan. *Clin. Chem.*, **1978**, 24, 1464.
- [19] D Jury; J Speed; P Dunn. *Clin.Chim. Acta*, **1985**, 148, 63-7.
- [20] C Cambiaso; D Collet-Cassart; M Lievens. *Clin. Chem.*, **1988**, 34(2), 416-8.
- [21] A Bernard; R Lauwerys. *Clin. Chem.Lab. Med.*, **1983**, 21(1), 25-30.
- [22] SC Tiu; SS Lee; MW Cheng. *Diabetes Care*, **1993**, 16(4), 616-20.
- [23] WL Roberts; CB Calcote; CB Cook; DL Gordon; ML Moore; S Moore; WD Scheer; BA Snazelle. *Clin.Chim. Acta*, **1998**, 273, 21-3.
- [24] AJ Bakker; A Bierma-Ram; H Keidel; H Syperda; A Zijlstra. *Clin. Chem.*, **2005**, 51(6), 1070-1.
- [25] Z Shihabi; J Konen; M O'connor. *Clin. Chem.*, **1991**, 37(5), 621-4.
- [26] D Jury; D Mikkelsen; P Dunn. *Clin. Chem.*, **1990**, 36(8), 1518-9.
- [27] AW Butch. *Clin. Chem.*, **2000**, 46(10), 1719-21.
- [28] L Paloheimo; M Pajari-Backas; E Pitkanen; L Melamies; R Rissanen. *J. Clin. Chem. Clin. Biochem.*, **1987**, 25(12), 889-92.
- [29] BK Kramer; U Jesse; KM Ress; RM Schmulling; T Risler. *Clin. Chem.*, **1987**, 33(4), 609-11.
- [30] MM Landgraf-Leurs; E Modi; K Horn; R Landgraf. *J. Clin. Chem. Clin. Biochem.*, **1987**, 25(10), 683-7.
- [31] H Thakkar; DJ Newman; P Holownia; CL Davey; CC Wang; J Lloyd; AR Craig; CP Price. *Clin.Chem.*, **1997**, 43(1), 109-13.
- [32] G Gotelli; M Mitchell. *Clin.Chem.*, **1985**, 31(6), 1085-6.
- [33] F Parant; M Moulisma; MC Gagnieu; G Lardet. *Ther.Drug Monit.*, **2005**, 27(4), 457-62.
- [34] J Ortega-Vinuesa; D Bastos-Gonzalez. *J. Biomat. Sci. Polym. E.*, **2001**, 12(4), 379-408.
- [35] J Molina-Bolivar; F Galisteo-Gonzalez. *Polym. Rev.*, **2005**, 45(1), 59-98.
- [36] CP Price; K Spencer; J Whicher. *Ann. Clin. Biochem.*, **1983**, 20, 1-14.
- [37] K Spencer; C Price. *Clin. Chim. Acta*, **1979**, 95(2), 263-76.
- [38] A Bakker. *Clin.Chem.*, **1988**, 34(1), 82-6.
- [39] J Lizana; K Hellsing. *Clin.Chem.*, **1974**, 20 (4), 415.
- [40] JM Bland; DG Altman. *Stat. Methods Med. Res.*, **1999**, 8(2), 135-60.