Microalbuminuria Analysis in Thai Patients with Diabetes and Hypertension Using Albumin Blue 580 Fluorescence Assay

Nuntra Suwantarat, M.D.*, Chatchawan Srisawat, M.D., Ph.D.*, Patarabutr Masaratana, M.D.*, Rungtip Soi-ampornkul, Ph.D.*, Sarawut Junnu, B.Sc.*, Weranuj Roubsanthisuk, M.D., Neelobol Neungton, M.D.***

**Department of Biochemistry, **Department of Internal Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*

ABSTRACT

Objective: To evaluate the performance of the fluorescence assay using albumin blue 580 for microalbuminuria, which is one of the early signs of renal diseases and an important cardiovascular risk factor for patients with diabetes and hypertension.

Methods: The fluorescence assay was tested for its precision and reliability by determining the intraassay and interassay coefficients of variation (CV). The correlation of the assay with the standard immunoturbidimetric assay (DCA 2000® microalbumin/creatinine reagent kit), which is one of the methods routinely used for microalbuminuria, was evaluated by quantitating the urinary albumin levels in 13 urine samples by both methods and the results were compared. The fluorescence assay was also used to detect the presence of microalbuminuria in 11 healthy subject, 11 patients with hypertension, and 10 patients with diabetes and hypertension.

Results: At the albumin concentrations of 5, 50, and 150 mg/L, the intraassay CVs of the fluorescence assay were 7.9, 4.4, and 3.5%, while the interassay CVs were 4.1, 8.0, and 0.4%, respectively. The fluorescence assay also showed a very good correlation with the standard immunoturbidimetric assay, with the intraclass correlation coefficient of 0.94 (0.81 to 0.98 at 95% confidence interval). When the assay was used to detect the presence of microalbuminuria (the excretion of 30-300 μg albumin/mg creatinine), it identified two out of 11 patients with hypertension (18%) and three out of 10 patients with both diabetes and hypertension (30%) having microalbuminuria whereas none of the healthy subjects had the condition. In addition, the presence of clinical albuminuria (the excretion of more than 300 μg albumin/mg creatinine) could also be identified in three patients with hypertension (27%) and one patient with both diabetes and hypertension (10%) respectively.

Conclusion: The fluorescence assay using albumin blue 580 was found to be precise and reliable and also showed a very good correlation with the standard immunoturbidimetric assay. In addition, the fluorescence assay is simple and the assay cost is much cheaper compared with the immunoturbidimetric measurement. Therefore, it could be another alternative method for microalbuminuria, particularly for most hypertensive or diabetic patients in Thailand, who can benefit from the detection of microalbuminuria but cannot afford regular tests.

Keywords: Correlation study; Diabetes; Fluorescence assay; Hypertension; Immunoturbidimetric assay;

Siriraj Med J 2006; 58: 1050-1053
E-journal: http://www.sirirajmedj.com

Microalbuminuria is defined as the excretion of 30-300 mg of albumin/24 hours, or 20-200 μg/min, or 30-300 μg albumin/mg creatinine in at least two out of three urine samples.[1, 2] It is recognized as one of the earliest indications of diabetic nephropathy which is a common and serious complication in diabetes with a prevalence of 30-40%,[3, 4] and a cardiovascular risk factor in patients with diabetes and hypertension.[1, 2, 5] Its incidences in patients with diabetes, hypertension, and nondiabetes-nonhypertension are 10-30%, 5-25%, and 5-10%, respectively.[2] Microalbuminuria is a key indicator of the need for intensified treatment with antihypertensive drugs in order to avoid renal complications. Angiotensin-converting enzyme inhibitors or angiotensin II antagonists could decrease urinary albumin excretion by lowering intraglomerular pressure, thus preventing persistent albuminuria and end-stage renal failure.[1-3, 5, 6] Therefore, it is recommended to check for the presence of microalbuminuria in type 2 diabetic patients at the time of diagnosis, as well as those with type 1 after having been diagnosed for 5 years. If the initial screening shows the absence of microalbuminuria, however, the test should be repeated annually.[1] Apart from diabetic patients, microalbuminuria is also common among hypertensive patients with left ventricular hypertrophy, overt cardiovascular symptoms,

Correspondence to: Chatchawan Srisawat
E-mail: sicss@mahidol.ac.th

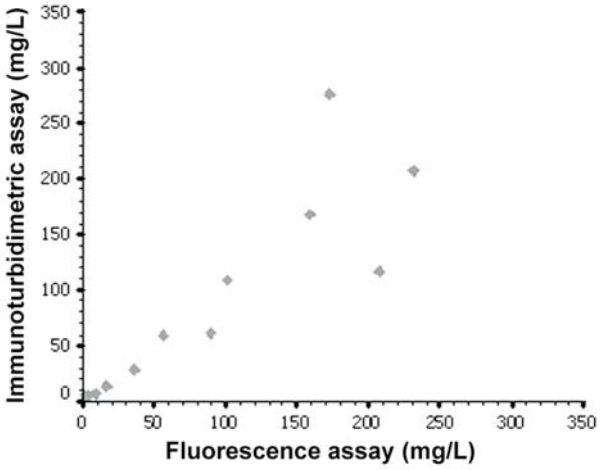

Fig 1. A bivariate plot of the assay results obtained from the fluorescence method, compared with those of the standard immunoturbidimetric method. The graph shows that the both assays have a very good correlation, with the intraclass correlation coefficient of 0.94 (0.81 to 0.98, 95% confidence interval).

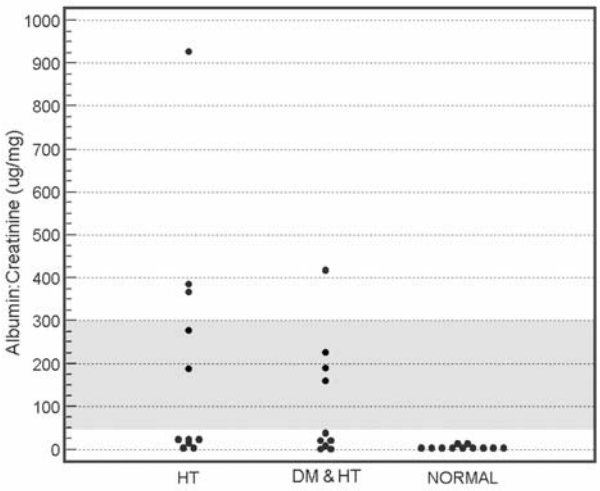

Fig 2. A scattergram of urinary albumin:creatinine (μg/mg) levels in the patients affected with hypertension (HT), both diabetes and hypertension (DM & HT), and the normal subjects. The levels corresponding to microalbuminuria is 30-300 μg albumin/mg creatinine as shown in the shaded area. The level of more than 300 μg albumin/mg creatinine is considered clinical albuminuria.

and subclinical evidences of atherosclerotic diseases (such as silent myocardial ischemia, increased carotid wall thickness, and ischemic white matter changes on cerebral imaging).[7] Therefore, the assay for microalbuminuria is included in the clinical practice guidelines for the management of essential hypertension.[7, 8]

There are several methods for microalbuminuria detection, which could be categorized into two main groups, i.e., qualitative and quantitative tests. The qualitative or semiquantitative detection, such as the Micral test, is somewhat inexpensive but less accurate. In contrast, the quantitative determinations such as immunoassays and high-performance liquid chromatography are accurate but somewhat complicated, and expensive. Recently, a novel fluorescence assay of microalbuminuria using a fluorescent dye, albumin blue 580, has been developed.[5, 9] The dye binds specifically to human albumin and the enhanced fluorescent signals of the dye-albumin complexes can be measured spectrofluorometrically. Besides the good precision and reliability of this fluorescence assay in quantitation of microalbuminuria,[5, 9] the quantitation procedures are simple and the assay cost is also relatively inexpensive compared with that of the immunoturbidimetric assay which is currently used in Siriraj Hospital. Thus, this fluorescence assay might prove to be useful as an alternative method that is inexpensive and more affordable, particularly, to the diabetic or hypertensive patients in Thailand, many of whom are poor and cannot be routinely evaluated for the presence of microalbuminuria due to the high cost of the test.

Therefore, in this study, the performance of the fluorescence assay using albumin blue 580 was evaluated in comparison with the standard immunoturbidimetric test using DCA 2000® microalbumin/creatinine reagent kit. In addition, the assay was also evaluated for its effectiveness in detecting the presence of microalbuminuria in the patients with diabetes and hypertension.

MATERIALS AND METHODS

Materials: Reagent A containing albumin blue 580 in 2-propanol (product No.05497) and human albumin (product No.05418) were obtained from Fluka, Switzerland. Other reagents [e.g., N-morpholino-propanesulfonic acid (MOPS), ethylenediaminetetraacetic acid (EDTA), propanol] were from Sigma, USA. DCA2000® microalbumin/creatinine reagent kit assay and DCA 2000® Analyzer were from Bayer, USA. The Shimadzu model RF-5000 spectrofluorometer (Kyoto, Japan) was used for spectrofluorometric analysis.

Urine samples collection and storage: Fresh random urine samples were collected and kept at - 20°C. The urines were obtained from the patients with hypertension alone (n=11), those with both diabetes and hypertension (n=10), and the healthy subjects (n=11). The subjects were selected based on the following inclusion criteria: a) the control subjects were currently healthy and had no previous history of chronic illnesses such as hypertension, diabetes and renal diseases; b) the patients with hypertension were those with blood pressure higher than 140/90 mmHg at the time of recruitment and currently treated with either antihypertensive medications or lifestyle modification; and, c) the patients with both diabetes and hypertension were recruited using the above criteria as the hypertensive patients and also affected with diabetes, which was being treated with either medications or lifestyle modification. The subjects who had the following conditions, i.e., menstruation, heavy exercise, pregnancy, vaginal discharge, plasma creatinine higher than 2 mg/dl, urine albumin concentration of more than 300 mg per 24 hours, fever, acute illness, hematuria, urinary tract infection, congestive heart failure, fasting blood sugar over 300 mg/dl, diabetic retinopathy, and diabetic nephropathy, were excluded from the study.

Albumin fluorescence assay protocol: The fluorescence assay was performed as previously described[9]. Briefly, two milliliters of Reagent A containing 71 μmol of albumin blue 580 in 2-propanol were mixed with 100 ml of Reagent B containing MOPS buffer pH 7.4 ± 0.2 (the buffer was prepared by mixing 3.0 g of MOPS free acid, 9.0 g MOPS sodium salt, 12.0 g sodium chloride,

1.0 g EDTA disodium salt, 900 ml of distilled water, and 100 ml of 2-propanol) to make the assay reagent, which was prepared fresh each day and kept in a closed glass bottle. To determine the albumin level in the urine, 0.5 ml of the urine sample was mixed with 2.5 ml of the assay reagent and the fluorescence intensity was measured immediately (or within 5 min) with a spectrofluorometer in a 1-cm standard fluorescence cuvette. The excitation and emission wavelengths were 600 and 630 nm, respectively. The calibration curve was generated using standard human albumin solutions at concentrations of 2, 10, 30, 100, and 200 mg/L, respectively. The curve was well approximated by the function $y = Ax/(1+Bx) + C$, where x was the concentration of albumin in the sample (mg/L), y was the relative intensity of fluorescence emission, and A, B and C were parameters obtained from a curve-fitting program (CurveExpert 1.34). The urine creatinine was determined spectrophotometrically by Taussky's method.[10] The microalbumin level was expressed in μg of albumin/mg of creatinine. The criteria for microalbuminuria were defined as the level of urinary albumin between 30-300 μg/mg of creatinine.

The albumin levels in 13 urine samples were also measured using the standard immunoturbidimetric method (DCA2000® microalbumin/creatinine reagent kit assay and DCA2000® Analyzer) and compared with the results from the fluorescence assay.

RESULTS

Three different samples containing human albumin at 5, 50, and 150 mg/L were measured in duplication and for two or more occasions using the fluorescence assay. The intraassay and interassay coefficients of variation (CV) were determined to evaluate the assay precision and reliability. The fluorescence assay was found to be precise and reliable as shown from the low intraassay CVs (7.9, 4.4, and 3.5%) and interassay CVs (4.1, 8.0, and 0.4%) at the albumin concentrations of 5, 50, and 150 mg/L, respectively.

To evaluate the correlation of the fluorescence assay with the standard immunoturbidimetric assay, 13 urines samples were analyzed by both methods and the results were compared. The fluorescence assay had a very high degree of correlation with the immunoturbidimetric measurement, with the intraclass correlation coefficient of 0.94 (0.81-0.98 at 95% confidence interval) as shown in Fig 1.

The fluorescence assay was then used to evaluate the urinary albumin levels in the healthy subjects and the patients. The median (and range) of urinary albumin/creatinine in the control group, the patients with hypertension, and the patients with both diabetes and hypertension, were 3.9 (1.26-13.58), 25.1 (1.01-926.00) and 31.57 (0.86-416.55) μg of albumin/mg of creatinine, respectively. From this study, none of the healthy subjects had the urinary albumin levels in the range of microalbuminuria (30-300 μg of albumin/mg of creatinine), whereas two out of 11 patients with hypertension (18%) and three out of 10 patients with both diabetes and hypertension (30%) were found to have the presence of microalbuminuria (Fig 2). In addition, the presence of clinical albuminuria (the excretion of albumin more than 300 μg/mg creatinine) could also be identified in three patients with hypertension (27%) and one patient with both diabetes and hypertension (10%), indicating the renal complications have already developed in some of these hypertensive and/or diabetic patients.

DISCUSSION

The performance of fluorescence assay using albumin blue 580 was evaluated in this study and the test was found to be quite precise and reliable, with the intraassay and interassay CVs below 10%. In addition, the fluorescence assay also correlated well with the standard immunoturbidimetric assay (the intraclass correlation coefficient of 0.94), suggesting that the assay was accurate and could be used for detection of microalbuminuria. When used to detect the presence of microalbuminuria, it could identify some of the hypertensive and/or diabetic patients with microalbuminuria, whereas none of the healthy subjects had the condition.

The fluorescence assay has several advantages over the routinely-used immunoturbidimetric method. The assay procedures are quite simple and the cost is much cheaper; the estimated cost of the fluorescence method is about 100 baht/test, compared to that of the immunoturbidimetric method which is about 400 baht/test. Because of its good precision, reliability, and a cheaper assay cost, the fluorescence assay could be another alternative method for detection of microalbuminuria; its simple procedures could make it more easily adopted into a routine service by small or community hospitals, and its cheaper cost should make the test more affordable for most hypertensive or diabetic patients in Thailand, who can benefit from the detection of microalbuminuria but cannot afford regular tests.

ACKNOWLEDGEMENTS

This work was supported by the Siriraj Grant for Research, Faculty of Medicine, Siriraj Hospital, Mahidol University. The authors greatly appreciated Mr.Suthipol Udompunturak for his help with the statistical analysis.

REFERENCES

1. Molitch ME, DeFronzo RA, Franz MJ. Nephropathy in diabetes. Diabetes Care 2004; 27: S79-83.
2. Monster TB, Janssen WM, de Jong PE, de Jong-van den Berg LT. The impact of antihypertensive drug groups on urinary albumin excretion in a non-diabetic population. Br J Clin Pharmacol 2002; 53: 31-6.
3. Comper WD, Osicka TM, Jerums G. High prevalence of immuno-unreactive intact albumin in urine of diabetic patients. Am J Kidney Dis 2003; 41: 336-42.
4. Comper WD, Osicka TM, Clark M, MacIsaac RJ, Jerums G. Earlier detection of microalbuminuria in diabetic patients using a new urinary albumin assay. Kidney Int 2004; 65: 1850-5.
5. Kessler MA, Meinitzer A, Petek W, Wolfbeis OS. Microalbuminuria and borderline-increased albumin excretion determined with a centrifugal analyzer and the Albumin Blue 580 fluorescence assay. Clin Chem 1997; 43: 996-1002.
6. Mogensen CE, Christensen CK, Vittinghus E. The stages in diabetic renal disease. With emphasis on the stage of incipient diabetic nephropathy. Diabetes 1983; 32: 64-78.
7. Donnelly R, Rea R. Microalbuminuria: how informative and reliable are individual measurements? J Hypertens 2003; 21: 1229-33.
8. Rosa TT, Palatini P. Clinical value of microalbuminuria in hypertension. J Hypertens 2000; 18: 645-54.
9. Kessler MA, Meinitzer A, Wolfbeis OS. Albumin blue 580 fluorescence assay for albumin. Anal Biochem 1997; 248: 180-2.
10. Bonsnes RW, Taussky HN. On the colorimetric determination of creatinine by Jaffe reaction. J Biol Chem 1945; 158: 581-91.

บทคัดย่อ

การวิเคราะห์ภาวะไมโครอัลบูมินนูเรีย (microalbuminuria) ในผู้ป่วยไทยที่เป็นโรคเบาหวานและความดันโลหิตสูงโดยวิธี albumin blue 580 fluorescence assay

นันตรา สุวันทารัตน์ พ.บ.*, ชัชวาลย์ ศรีสวัสดิ์ พ.บ., ปร.ด.*, ภัทรบุตร มาศรัตน พ.บ.*, รุ่งทิพย์ สร้อยอัมพรกุล ปร.ด.*, ศราวุธ จั่นหนู วท.บ.*, วีรนุช รอบสันติสุข พ.บ., นิโลบล เนื่องตัน พ.บ.***

**ภาควิชาชีวเคมี, **ภาควิชาอายุรศาสตร์, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กทม. 10700, ประเทศไทย.*

วัตถุประสงค์: เพื่อประเมินผลการวิเคราะห์การตรวจหาภาวะไมโครอัลบูมินนูเรีย (microalbuminuria) ด้วยวิธีฟลูออเรสเซนซ์โดยใช้ albumin blue 580 ซึ่งภาวะไมโครอัลบูมินนูเรียนี้สามารถบ่งชี้ถึงการทำงานของไตที่เสื่อมในระยะเริ่มแรกและเป็นปัจจัยเสี่ยงที่สำคัญของโรคหลอดเลือดหัวใจตีบ โดยเฉพาะในผู้ป่วยโรคเบาหวานและโรคความดันโลหิตสูง

วิธีการ: ประเมินความแม่นยำและความเชื่อถือได้ของวิธีฟลูออเรสเซนซ์โดยทดสอบหาค่าสัมประสิทธิ์การแปรผันภายในและระหว่างการวิเคราะห์ (intraassay และ interassay coefficient of variation) รวมทั้งหาความสัมพันธ์ระหว่างวิธีฟลูออเรสเซนซ์กับวิธี immunoturbidimetric measurement (ด้วย DCA 2000® microalbumin/creatinine reagent kit) ซึ่งเป็นวิธีมาตรฐานวิธีหนึ่งที่ใช้ในการตรวจหาภาวะนี้ โดยทำการทดสอบและเปรียบเทียบระดับของอัลบูมินในปัสสาวะที่ได้โดยวิธีทั้งสองจากปัสสาวะของผู้ป่วยจำนวน 13 ตัวอย่าง หลังจากนั้นได้ทดสอบการใช้วิธีฟลูออเรสเซนซ์นี้ในการวิเคราะห์ปัสสาวะในคนปกติจำนวน 11 ราย, ผู้ป่วยโรคความดันโลหิตสูงจำนวน 11 ราย และผู้ป่วยโรคเบาหวานที่มีภาวะความดันโลหิตสูงร่วมด้วย จำนวน 10 ราย

ผลการศึกษา: ค่าสัมประสิทธิ์การแปรผันภายในการวิเคราะห์ของวิธีฟลูออเรสเซนซ์ที่ระดับความเข้มข้นของอัลบูมินในปัสสาวะที่ 5, 50 และ 150 มก/ล เท่ากับ 7.9, 4.4 และ 3.5% ขณะที่ค่าสัมประสิทธิ์การแปรผันระหว่างการวิเคราะห์เท่ากับ 4.1, 8.0 และ 0.4% ตามลำดับ นอกจากนี้ผลการศึกษายังพบว่าวิธีฟลูออเรสเซนซ์นี้มีความสัมพันธ์ดีมากกับวิธีมาตรฐาน โดยมี intraclass correlation coefficient เท่ากับ 0.94 (0.81- 0.98 ที่ช่วงความเชื่อมั่นร้อยละ 95) และเมื่อใช้วิธีฟลูออเรสเซนซ์ในการตรวจหาภาวะไมโครอัลบูมินนูเรีย (ระดับอัลบูมินในปัสสาวะเท่ากับ 30-300 มคก/มก.ครีอะตินีน) พบว่า พบภาวะนี้ในผู้ป่วย 2 รายจาก 11 ราย (18%) ในกลุ่มผู้ป่วยความดันโลหิตสูง และ 3 รายจาก 10 ราย (30%) ในกลุ่มผู้ป่วยโรคเบาหวานที่มีความดันโลหิตสูงร่วมด้วย แต่ไม่พบภาวะนี้ในกลุ่มคนปกติ นอกจากนี้ยังพบภาวะ clinical albuminuria (ระดับอัลบูมินในปัสสาวะมากกว่า 300 มคก/มก.ครีอาตินีน) ในผู้ป่วย 3 ราย (27%) ในกลุ่มผู้ป่วยความดันโลหิตสูง และ 1 ราย (10%) ในกลุ่มผู้ป่วยโรคเบาหวานที่มีความดันโลหิตสูง ตามลำดับ

สรุป: การวิเคราะห์ไมโครอัลบูมินนูเรียในปัสสาวะด้วยวิธีฟลูออเรสเซนซ์โดยใช้ albumin blue 580 พบว่ามีความแม่นยำ เชื่อถือได้สูง และมีสัมพันธ์ดีมากกับวิธี immunoturbidimetric ซึ่งเป็นวิธีมาตรฐานวิธีหนึ่งสำหรับภาวะไมโครอัลบูมินนูเรีย นอกจากนี้วิธีฟลูออเรสเซนซ์ยังมีค่าใช้จ่ายในการวิเคราะห์ที่ถูกกว่าและขั้นตอนการวิเคราะห์ที่ง่ายกว่า ดังนั้นการทดสอบนี้น่าจะมีความเหมาะสมสำหรับเป็นทางเลือกหนึ่งในการตรวจหาภาวะไมโครอัลบูมินนูเรีย โดยเฉพาะในผู้ป่วยโรคเบาหวานและความดันโลหิตสูงในประเทศไทยส่วนใหญ่ ซึ่งสามารถได้รับประโยชน์จากการตรวจหาภาวะนี้แต่อาจจะไม่สามารถได้รับการตรวจได้อย่างสม่ำเสมอเนื่องจากปัญหาค่าใช้จ่ายในการตรวจ