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Measurement of Urine Albumin Levels With A Monoclonal Antibody Based in-House Elisa

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Abstract: Microalbuminuria is considered to be an early indicator of diabetic nephropathy. We have previously reported the establishment of A-HAlb/98 hybridoma secreting monoclonal antibody that specifically recognizes human serum albumin (HSA). In this report, an in-house enzyme-linked immunosorbent assay (ELISA) was developed using this antibody for the measurement of urinary albumin (UA) and UA was examined in 24 different samples. A-HAlb/98 was used as the capture antibody by immobilizing onto microtitre plates in ELISA format. HSA-specific polyclonal rabbit immunoglobulin was labelled with biotin and then used as the tracer antibody. The dynamic assay range of ELISA for albumin was established to be 10-1000 ng/ml, and the albumin levels determined by ELISA were well correlated (r = 0.8831) with those obtained by the liquid-phase immunoprecipitation method (The Orion Diagnostica TURBOX® U-albumin assay). The ability of this in-house ELISA to quantitate UA in nanogram ranges makes it a favourable candidate for utilization in diagnostic applications as well as in research studies.

Key Words: Human albumin, Monoclonal antibody, Polyclonal antibody, ELISA

Introduction

In healthy humans albumin is excreted in small amounts in the urine. However, in diabetic nephropathy the urinary albumin levels are increased (1). It was also reported that faecal albumin levels were increased in patients with colorectal cancers (2). The composition of saliva is altered in patients with diabetes, with increased secretion of calcium and immunoglobulin (3). It has been suggested that this might result from an increase in microvascular permeability (3). Although salivary acinar cells do not secrete albumin, it might enter the saliva (4) following leakage from surrounding blood vessels because the molecular weight of albumin is lower than that of immunoglobulin. Albumin concentrations in the cerebrospinal fluid (CSF) were also reported to be at low levels ranging from 100 to 800 µg per ml (5) and the IgG levels, IgG/albumin ratios and IgG indexes were significantly increased in the CSF of multiple sclerosis (MS) patients compared to those of non-MS controls (6).

In order to measure albumin as a predictive marker in some situations such as that mentioned above, a highly sensitive and specific method for determining low concentrations of albumin is needed. The aim of the present study was to develop an ELISA for quantifying human albumin in urine samples.

Materials and Methods

Human albumin. Human serum albumin (HSA, approx. 99% (agarose electrophoresis), essentially fatty acid free (approx. 0.005%), essentially globulin free, lyophilized powder) was purchased from Sigma Chemical Co. St. Louis, MO, USA.

Monoclonal antibody. A-HAlb/98 hybridoma cells were used as the source of IgG1 monoclonal antibodies specific for HSA (7). Monoclonal antibodies were purified from ascites using a protein G affinity column (8). Protein contents were measured with the Bradford method (9) using bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) as the standard.

Polyclonal antibody. An albino rabbit was immunized with 200 µl of antigen (200 µg of HSA) in 200 µl of complete Freund's adjuvant followed by a second injection in 200 µl of incomplete Freund's adjuvant at 30-day intervals. Two booster injections, with antigen only, were performed at 20-day intervals. In order to obtain serum samples, the rabbit was bled from the central auricle artery 10 days after the last injection. All subcutaneous or intradermal injections and blood collections were performed after anaesthetizing the rabbit with an intramuscular injection of ketamin/xylazine cocktail. Anti-HSA polyclonal antibody containing rabbit IgG was

purified from the serum using a protein A affinity column (8). The biotinylation of IgG was performed as described elsewhere (10). A working concentration of biotinylated rabbit IgG was prepared by diluting to 1:10,000 in PBS containing 5% foetal calf serum (FCS, Seromed, Biochrom KG Leonarenstr. 2-6 D-12247 Berlin, Germany).

Urine samples. Twenty-four different urine samples were collected and frozen at – 74 °C until the day of study.

Enzyme-linked immunoabsorbent assay (ELISA). High binding capacity ELISA plates (Costar, No: 3590, Corning Incorporated, Corning, NY 14831, USA) were coated with 100 µl of ascite-purified A-HAlb/98 at 1 µg/ml in 50 mM carbonate-bicarbonate buffer pH 9.6 by incubating at 4 °C overnight. After several washings with distilled water, the wells were blocked with 300 µl of PBS containing 1% BSA by incubating at 4 °C overnight followed by washing three times with PBS containing 0.05% Tween 20 (PBS-T). To each well was added 100 µl of 1/100 diluted samples or standards, followed by incubation at 37 °C for 1 h. After incubation, the plates were washed three times with PBS-T and then incubated with 100 µl of biotinylated rabbit anti-HSA polyclonal IgG at 37 °C for 1 h. After incubation, the plates were washed three times with PBS-T and then incubated with 100 µl of streptavidine-peroxidase (Sigma Chemical Co. St. Louis, MO, USA) at 37 °C for 30 min. After washing, the reaction was revealed with 3,3',5,5' tetramethylbenzidine (TMB) solution, formulated in our laboratory, for 30 min at room temperature. After stopping the reaction with 50 µl of 2M H_2SO_4, the plates were read with an ELISA reader (LP400, Diagnostics Pasteur, France) at 450 nm with or without the reference against 620 nm.

Liquid-phase immunoprecipitation assay. The Orion Diagnostica TURBOX® U-albumin assay (Orion Corporation Orion Diagnostica, Espoo, Finland), which is a commercially available liquid-phase immunoprecipitation assay with nephelometric end-point detection, was used to measure albumin levels in 24 different urine samples for comparison. The kit was used according to the manufacturer's instructions.

Statistical analysis. Comparison of the data between the various methods was performed with correlation analysis. $P < 0.05$ was considered significant. Intra- and inter-assay coefficients of variation (CV) were measured according to the following formula:

$$CV = S \bullet \frac{100\%}{\overline{X}} = \sqrt{\frac{\Sigma (X - \overline{X})^2}{N - 1}} \bullet \frac{100\%}{\overline{X}}$$

where

S = standard deviation of optical density (OD) readings,

$\overline{X}$ = mean of OD readings,

X = individual OD readings,

N = number of readings (wells)

Results

The in-house human albumin ELISA demonstrated a sensitivity level of less than 10 ng/ml (Figure 1). The dynamic analytical measuring range was between 10 and 1000 ng per ml. When four calibration points (0, 10, 100, and 1000 ng/ml) were plotted, a significant correlation ($p = 0.027$, $r = 0.972$) between albumin levels and OD450 nm values was observed (Figure 1). Albumin concentrations higher than 1000 ng/ml produced overflow values at OD450 nm.

The quality control of the in-house albumin ELISA was performed by analysing its intra- and inter-assay CV. When human albumin at various concentrations was tested, the intra- and inter-assay CV values were 1.8-7.4% and 6.2-9.3% respectively (Table).

Albumin levels of 24 different urine samples were measured with both the in-house ELISA and a commercial microalbuminuria kit, TURBOX®, which is designed to quantify albumin levels between 5 and 160 mg/l. There was a significant correlation ($p < 0.0001$, $r = 0.883$) between the albumin levels measured with two different assay types (Figure 2).

Discussion

Quantification of albumin in microgram levels has been studied in various body fluids as well as tissues either for diagnostic or investigational purposes (2,3,6). These concerns fall in a very wide clinical range and the investigational spectra have to rely on a simple, sensitive and cheap measuring method, especially in the case of countries with limited health budgets.

The detection of microalbuminuria (mAlbU) has been reported to indicate a high probability of damage to the

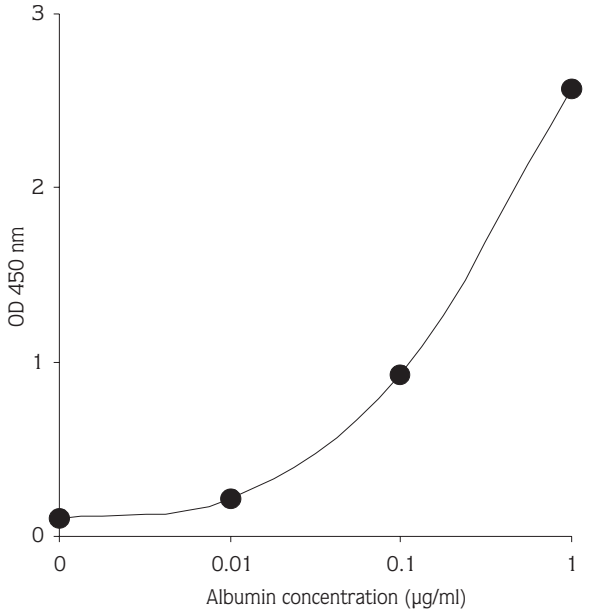

Figure 1. Enzyme-linked immunosorbent assay of human albumin. A-HAlb/98 mAb-immobilized microtitre wells were incubated with varying amounts of human albumin as indicated (see text for details). Binding was detected with biotinylated rabbit anti-human albumin second antibody/streptavidine-HRP conjugate and TMB substrate. A prominent correlation (r = 0.9721, p = 0.027) was observed between the albumin concentration and the optical density. Results are the mean of quadruplicate observations.

Table. Intra- and inter-assay coefficients of variation (CV) of albumin ELISA.

Standard (ng/ml)	Intra-assay CV (%) (n = 5)	Inter-assay CV (%) (n = 4)
1000	1.8	8.6
100	2.1	6.2
10	7.4	9.3

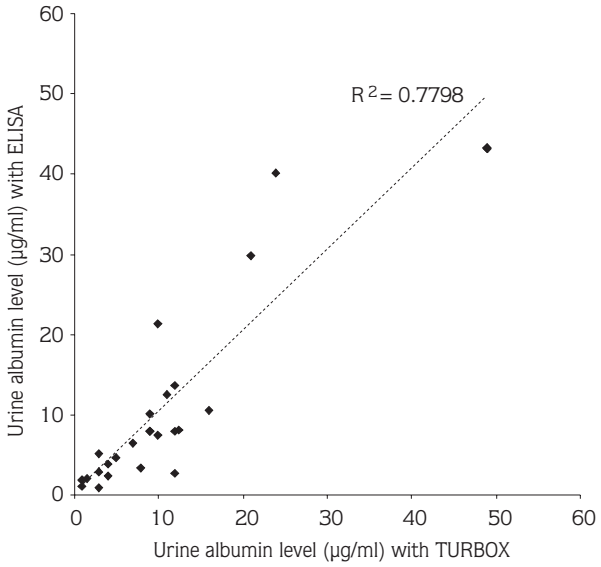

Figure 2. Comparison of the in-house ELISA and TURBOX® for human albumin levels in urine samples. Twenty-four urine samples were measured for their albumin contents with the in-house ELISA and a commercial kit, TURBOX®, which is a liquid–phase immunoprecipitation assay with nephelometric end-point detection. For the in-house ELISA, urine samples were first diluted to 1/100 and then OD values were plotted against the standard curve to find out albumin contents. The results were multiplied by the dilution factor and then converted to µg/ml. There was a significant correlation (r = 0.8831, p < 0.0001) between the two different types of assay.

glomerular filtration capacity of the kidneys and is of great screening and/or diagnostic interest (11,12). Diabetes mellitus is the leading cause of end-stage renal diseases and routine screenings for mAlbU is a well-established recommendation for type I and type II diabetes in recent guidelines (11). With regard to the time process of diabetes, it is obvious that patients have to be followed-up for many years and albuminuria develops within the first few years after the onset of diabetes (12).

The important role of screening and quantifying mAlbU as a risk factor for cardiovascular disease and vascular dysfunction is also emphasized in recent reports (13-18) and mAlbU has been established as a risk factor for developing left ventricular hypertrophy (19). Beyond these major clinical concerns its role as a scoring system in critically ill patients admitted to intensive care units with a wide range of diagnoses (20) and as a prognostic factor in patients with acute respiratory or multi-organ failure has been proposed (21). The quantifying of mAlbU has also been studied as an objective marker for the outcome effects of various therapies (22-26) in different patient populations. Its role as a predictor of pre-eclampsia in pregnant hypertensive women (27) or of gestational age at delivery (28) has also been documented. Levels of mAlbU have also been related to the tumour burden in non-Hodgkin's lymphoma patients (29) and in patients with lung cancer (30). Microalbuminuria is reported to be a marker for nephron development in various age groups (31).

Although timed sampling (12-24 h collection of urine) is recommended as the sampling method (11), random urine samples have been also documented as a reliable sampling method for the quantification of mAlbU (32). In

the determination of the level of mAlbU, several methods have been employed in clinical and intervention studies and it has been shown that there could be discordance among various methods (33). Therefore, the method used for quantification should be simple and reliable and should have a high sensitivity in measuring the microalbumin level (11), especially in a range appropriate to that demarcating normal from microalbuminuric patients. Because of the possible therapeutic decisions with regard to treatment or follow-up, it is essential that accurate and reproducible methodologies with high sensitivity be employed. Otherwise misclassifications could lead to incorrect decisions.

The results of our mAb-based in-house ELISA display a quite sensitive level for the measurement of microalbuminuria. When the human serum albumin of known concentrations was used for the validation of our in-house assay, we obtained a quite favourable and significant correlation ($r = 0.972$, $p = 0.027$) for the tested calibration points. The in-house assay displayed overflow values at albumin concentrations greater than 1000 ng/ml, corresponding to the upper detection limit. In respect to the lower analytical limit, 10 ng/ml could be concluded to be the sensitivity of our in-house ELISA. We obtained a dynamic analytical measuring range of 10 to 1000 ng/ml, a range appropriate to that demarcating normal from microalbuminuric patients. For the quality control of the mAb based in-house assay, three different concentrations of standard human albumin were tested. Data obtained on intra- and inter-assay CV precision were analysed. Even at the lowest concentration of albumin (10 ng/ml) we obtained intra- and inter-assay CV of less than 10%. For all of the tested concentrations of standard human albumin the intra-and inter-assay CV showed dispersion indices with values of less than 10%, corresponding to quite satisfactor levels.

The in-house mAb based ELISA was compared with a commercially available kit, the TURBOX® microalbuminuria assay, which is a liquid-phase immunoprecipitation method with nephelometric end-point detection. The TURBOX® microalbuminuria assay is a commonly used system for both screening and diagnostic purposes in routine laboratories. All of the 24 void samples were tested with both the in-house ELISA and the TURBOX®. The results of the albumin levels measured with our in-house ELISA and the TURBOX® displayed a significant correlation ($r = 0.8831$, $p < 0.0001$). However, the in-house ELISA was detected to be sensitive beyond the minimum measuring level given for the TURBOX®. It can be concluded that in practical terms the mAb-based in-house ELISA appears to be more suitable and sensitive for mAlbU quantification than the commercially available liquid-phase immunoprecipitation assay.

The in-house ELISA seemed to be more advantageous because of its reliability and low cost compared to the commercially available kit. In addition, it may be promising for routine or investigational analysis of albumin at low concentrations in various body fluids. In summary, the present results suggest that the in-house mAb-based ELISA is a reliable and precise method for determining microalbuminuria.

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References

1. Fisher BM, Lamey PJ, Sweeney D, Beeley JA, Spooner RJ, Frier BM. Salivary secretion of albumin in type 1 (insulin-dependent) diabetes. Diabetes Res Clin Pract 11: 117-119, 1991.
2. Nakayama T, Yasuoka H, Kishino T, Ohguchi H, Takada M. ELISA for occult faecal albumin. Lancet 13: 1368-1369, 1987.
3. Marder M, Abelson DC, Mandel ID. Salivary alterations in diabetes mellitus. J Periodontol 46: 567-569, 1975.
4. Sweeney D, Beeley JA. An enzyme-linked immunoassay for human salivary albumin. Arch Oral Biol 35: 741-746, 1990.
5. Schwerer B, Bach M, Bernheimer H. ELISA for determination of albumin in the nanogram range: assay in cerebrospinal fluid and comparison with radial immunodiffusion. Clin Chim Acta 163: 237-244, 1987.

6. Mehta PD, Mehta SP. Quantitation of IgG and albumin in CSF and serum from multiple sclorosis patients by enzyme-linked immunosorbent assay. Acta Neurol Scand 75: 125-129, 1987.

7. Aybay C, İmir T, Kayhan B. A competitive enzyme-linked immunosorbent assay (ELISA) for determination of human albumin in the nanogram range. Gazi Medical Journal 10: 63-67, 1999.

8. Aybay C, İmir T. Development of a rapid, single-step procedure using protein G affinity chromatography to deplete fetal calf serum (FCS) of its IgG and to isolate murine IgG1 monoclonal antibodies from supernatants of hybridoma cells. J Immunol Methods 233: 77, 2000.

9. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.

10. Wisdom GB. Non-radioactive antibody probes. Monoclonal Antibodies (Eds. A. Shepherd and C. Dean), Oxford University Press, Oxford, 2000, pp. 237-246.

11. Sacks DB, Bruns DE, Goldstein DE, Maclaren NK, McDonald JM, Parrott M. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Clin Chem 48: 436-472, 2002.

12. Sosenko JM, Hu D, Welty T, Howard BV, Lee E, Robbins DC. Albuminuria in recent-onset type 2 diabetes: the strong heart study. Diabetes Care 25: 1078-1084, 2002.

13. Grandi AM, Santillo R, Bertolini A, Imperiale D, Broggi R, Colombo S, Selva E, Jessula A, Guasti L, Venco A. Microalbuminuria as a marker of preclinical diastolic dysfunction in never-treated essential hypertensives. Am J Hypertens 14: 644–648, 2001.

14. Garg JP, Bakris GL. Microalbuminuria: marker of vascular dysfunction, risk factor for cardiovascular disease. Vasc Med 7: 35-43, 2002.

15. Diercks GF, Stroes ES, Boven AJ, Roon AM, Hillege HL, Jong PE, Smit AJ, Gans RO, Crijns HJ, Rabelink TJ, Gilst WH. Urinary albumin excretion is related to cardiovascular risk indicators, not to flow-mediated vasodilation, in apparently healthy subjects. Atherosclerosis 163: 121–126, 2002.

16. Ruilope LM. Kidney dysfunction: a sensitive predictor of cardiovascular risk. Am J Hypertens 14: 213S–217S, 2001.

17. Brischetto R, Leonardi V, Amore MG, Corno C, Leotta S, Lizzio G, Bonsignore L, Calcara G. Significance of microalbuminuria in atherosclerotic vascular disease. Arch Gerontol Geriatr Suppl 5: 173-177, 1996.

18. Tomiyama H, Doba N, Kushiro T, Yamashita M, Kanmatsuse K, Kajiwara N, Yoshida H, Hinohara S. The relationship of hyperinsulinemic state to left ventricular hypertrophy, microalbuminuria, and physical fitness in borderline and mild hypertension. Am J Hypertens 10: 587-591, 1997.

19. Tomura S, Kawada K, Saito K, Lin YL, Endou K, Hirano C, Yanagi H, Tsuchiya S, Shiba K. Prevalence of microalbuminuria and relationship to the risk of cardiovascular disease in the Japanese population. Am J Nephrol 19: 13-20, 1999.

20. MacKinnon KL, Molnar Z, Lowe D, Watson ID, Shearer E. Use of microalbuminuria as a predictor of outcome in critically ill patients. Br J Anaesth 84: 239-241, 2000.

21. Abid O, Sun Q, Sugimoto K, Mercan D, Vincent JL. Predictive value of microalbumin in medical ICU patients: results of a pilot study. Chest 120: 1984-1988, 2001.

22. Turner R, Holman R, Stratton I, Cull C, Frighi V, Manley S, Matthews D, Neil A, McElroy H, Kohner E, Fox C, Hadden D, Wright D. Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38. UK Prospective Diabetes Study Group. BMJ 317: 703-713, 1998.

23. Skowsky WR, Siddiqui T, Hodgetts D, Lambrou FH, Stewart MW, Foster MT. A pilot study of chronic recombinant interferon-alfa 2a for diabetic proliferative retinopathy: metabolic effects and opthalmologic effects. J Diabetes Complications 10: 94-99, 1996.

24. Steindel BS, Roe TR, Costin G, Carlson M, Kaufman FR. Continuous subcutaneous insulin infusion (CSII) in children and adolescents with chronic poorly controlled type I diabetes mellitus. Diabetes Res Clin Pract 27: 199-204, 1995.

25. Maislos M, Weisman D, Sherf M. Western Negev mobile diabetes care program: a model for interdisciplinary diabetes care in a semi-rural setting. Acta Diabetol 39: 49-53, 2002.

26. Krespi PG, Makris TK, Hatzizacharias AN, Triposkiadis P, Tsoukala C, Kyriaki D, Votteas V, Kyriakidis M. Moxonidine effect on microalbuminuria, thrombomodulin, and plasminogen activator inhibitor-1 levels in patients with essential hypertension. Cardiovasc Drugs Ther 12: 463-467, 1998.

27. Paternoster DM, Stella A, Mussap M, Plebani M, Gambaro G, Grella PV. Predictive markers of pre-eclampsia in hypertensive disorders of pregnancy. Int J Gynaecol Obstet 66: 237-243, 1999.

28. Perry IJ, Gosling P, Sanghera K, Churchill D, Luesley DM, Beevers DG. Urinary microalbumin excretion in early pregnancy and gestational age at delivery. BMJ 307: 420-421, 1993.

29. Pedersen LM, Sorensen PG. Clinical significance of urinary albumin excretion in patients with non-Hodgkin's lymphoma. Br J Haematol 107: 889-891, 1999.

30. Pedersen LM, Milman N. Microalbuminuria in patients with lung cancer. Eur J Cancer 34: 76-80, 1998.

31. Hua MJ, Kun HY, Jie CS, Yun NZ, De WQ, Yang Z. Urinary microalbumin and retinol-binding protein assay for verifying children's nephron development and maturation. Clin Chim Acta 264: 127-132, 1997.

32. Assadi FK. Quantitation of microalbuminuria using random urine samples. Pediatr Nephrol 17: 107-110, 2002.

33. Roberts WL, Calcote CB, Cook CB, Gordon DL, Moore ML, Moore S, Scheer WD, Snazelle BA. Comparison of four commercial urinary albumin (microalbumin) methods: implications for detecting diabetic nephropathy using random urine specimens. Clin Chim Acta 273: 21-33, 1998.