Performance Characteristics of an HPLC Method for Urinary Albumin

William E. Owen[1], William L. Roberts[2]

[1]ARUP Institute for Clinical and Experimental Pathology Salt Lake City, Utah

[2]Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, Utah

Abstract

Microalbuminuria is a marker of early diabetic nephropathy and cardiovascular risk. Immunoassays for urinary albumin can underestimate the amount of intact albumin present in urine. The purpose of this study was to evaluate a new urinary albumin assay that employs size exclusion HPLC with UV detection to quantify urinary albumin. We determined the limit of detection (LOD), linearity, imprecision, comparison to an immunoturbidimetric assay (ITA), and pediatric and adult reference intervals on random urine collections. All subjects for reference interval studies were apparently healthy and none were taking prescription medications other than oral contraceptives. Boys and girls ranged from 7 to 17 years of age and 20 boys and 20 girls from each of these years of life were included. The LOD was 3.4 mg/L. The assay was linear from 4 to 240 mg/L. Within run imprecision was 4.9, 0.9, and 0.7% at 16, 89, and 206 mg/L, respectively. Total imprecision was 8.4, 3.1, and 2.9% at 16, 89, and 206 mg/L, respectively. Passing-Bablok analysis of HPLC versus ITA for 101 samples with albumin/creatinine ratios by ITA of 20 to 200 mg/g showed a positive proportional bias with a slope of 1.77, an intercept of 2.2 and r = 0.83. For 103 samples with albumin/creatinine ratios by ITA from 200 to 1000 mg/g, the slope was 1.33, the intercept was 20 and r = 0.87. Non-parametric reference intervals for both the ITA and HPLC methods are shown in the Table 2. The HPLC assay for total urinary albumin shows acceptable performance and quantifies albumin species not detected by immunoassay. Our studies of apparently healthy children and adults suggest the need for separate male and female and pediatric and adult reference intervals. Further outcome studies are necessary to determine how to best interpret urinary albumin measurements made by HPLC.

Introduction

Microalbuminuria (MA) is defined as the excretion of albumin in urine at levels of 20-200 ug/min or 30-300 mg/24hr for timed collections and 30-300 mg/g creatinine for random or spot urine samples. In type 1 and type 2 diabetes mellitus, MA is considered the earliest marker of diabetic nephropathy. Type 1 diabetics have been shown to develop nephropathy in >30% of cases. Approximately 25% of patients with type 2 diabetes mellitus have been shown to develop nephropathy 5-10 years after diagnosis. Diabetic nephropathy is the most common causes of end-stage renal disease in the US and Europe. The ability to detect MA early is clinically important because of the potential to initiate use of anti-angiotensin therapy in order to stop or slow the progression of diabetic nephropathy. In addition, MA has been shown to be a marker for increased risk of cardiovascular morbidity and mortality in both diabetic and non-diabetic patients.[1,2,3]

The ability to measure accurately and precisely the low concentrations of albumin in urine associated with MA has been facilitated by the development of automated immunoassay methods. Previously we have studied the performance of some of these commercially available assays. A series of recent studies have shown that current immunoassay methods may underestimate the amount of intact albumin in urine from diabetic subjects, especially at excretion rates less than 100 ug/min.[4-8] These studies have compared various immunoassay methods to a new HPLC assay that uses size exclusion chromatography. This method has recently received FDA clearance for the measurement of albumin in urine. The purpose of our study was to evaluate the analytic performance of this new assay and compare it to a conventional microalbumin immunoassay in normal and diabetic subjects. The need for robust reference intervals for both children and adults for this assay was a primary focus for this study.

Methods

- Wako Microalbumin B ITA assay (Wako Diagnostics, Richmond, VA) run on a Roche Modular P (Roche Diagnostics, Indianapolis, IN). This was used as the comparison method for our studies.
- Accumin direct total intact albumin by HPLC kit (AusAm Biotechnologies Inc., New York, NY) run on an Agilent/HP 1100 (Agilent Technologies, Los Angeles, CA) instrument.
- Two immunonephelometric assays (INA) for albumin were used. One on the BN II nephelometer using the manufacturer's reagent (Dade Behring, Deerfield, IL), and the other on the IMMAGE nephelometer using manufacturer's reagents (Beckman Coulter, Brea, CA).
- An immunochemiluminometric assay (ICMA) for albumin run on an IMMULITE 2000 instrument (Diagnostic Products Corporation, Los Angeles, CA) using manufacturer's reagent.
- Urinary creatinine testing by the Jaffe method on a Roche Modular P instrument using the manufacturer's reagent (Roche Diagnostics, Indianapolis, IN).

Procedures

The (LOD) was determined by assaying 10 replicate injections of the mobile phase buffer and 3 replicate injections of the low calibrator provided in the Accumin kit. The maximum amplitude from the buffer injections indicating the background noise level for the assay was compared to the average amplitude of the low calibrator, which had a concentration of 9.41mg/L. From the relationship of amplitude to albumin, a concentration of albumin equivalent to five times the background noise was determined to be the LOD. Linearity studies were performed using dilute urine from a healthy male volunteer (low pool) and supplementing with human serum albumin (Sigma, St. Louis, MO) to create a high pool. The high pool was diluted with the low pool to produce the following final concentrations of the high pool: 0%, 2.5%, 5%, 10%, 25%, 50%, 75% and 100%. These samples were analyzed in duplicate. Materials for imprecision testing were prepared by taking a dilute urine sample from a healthy volunteer and supplementing it with human serum albumin to produce three concentrations of albumin. Each imprecision pool was aliquotted and frozen at -70 °C until it was analyzed. Sufficient aliquots were prepared to perform two runs in duplicate per day for 10 days, for a total of 40 replicates per pool.

Method comparison samples with moderate albumin creatinine ratios (ACR) defined as 20 to 200 mg/g (n=101), and high ACR samples defined as 200 to 1000 mg/g (n=103) were retrieved from our patient sample frozen storage at -20 °C. They were then de-identified, and placed at -70 °C until time of testing. Before analyzing for albumin by HPLC, these samples were thawed, vortexed gently to mix and centrifuged at 3000g for 10 min. This albumin result was used to calculate the ACR using the original creatinine result. Adult reference samples were collected from apparently healthy volunteer subjects working at our company. Potential reference subjects were excluded for the following reasons: diabetes mellitus, hypertension, prescription medications except oral contraceptives, urinary tract infection in the last month, and females subjects who were menstruating. Our reference subjects included 80 males and 80 females, and ranged in age from 19 to 66 years. The samples were first or second morning void samples. Aliquots were frozen at -70 °C until time of analysis. Since our reference study subjects live at moderate altitude (1370 m) we arranged for reference samples to be collected from healthy volunteers living at sea level to account for any effect of altitude. The sea level samples were shipped frozen to our facility from their collection point. These samples were chiefly from young adults and included 66 females and 54 males with an age range from 18 to 26 years with one subject of 46 years. They were stored at -70 °C until time of testing. Our pediatric reference samples were random collections from healthy children who were on no prescription medications and included 20 male and 20 female subjects for each year of life from 7 to 17 for a total of 440 samples. All studies using samples from human subjects were approved by the Institutional Review Board of the University of Utah Health Sciences Center (Salt Lake City, UT). Linear regression and the general F-test for significance were performed using CBstat software version 5.0.0 (Kristian Linnet, Risskov, Denmark). EP Evaluator Release 5 software (D.G. Rhoads Associates Inc., Kennett Square, PA) was used for evaluation of linearity, imprecision, Passing-Bablok regression, calculation of r, and reference interval estimation including Z-test for partitioning by age and gender. Slope and intercept statistics for Passing-Bablok analysis are given as median with the 95% confidence interval in parentheses. Reference intervals were determined non-parametrically after exclusion of outliers as outlined in the NCCLS C28-A document.

Table 1. Summary of Imprecision Study Results

Pool Mean (mg/L)	Within-run CV (%)	Between Day CV (%)	Total CV (%)
15.9	4.9	5.7	8.4
89.3	0.9	3.0	3.1
205.7	0.7	2.7	2.9

Table 2. Non-parametric Reference Intervals for Urinary Albumin

Group	n	ITA reference interval (mg/g creatinine)	90% CI for ITA upper reference limit (mg/g creatinine)	HPLC reference interval (mg/g creatinine)	90% CI for HPLC upper reference limit (mg/g creatinine)	ITA reference interval (mg/L)	90% CI for ITA upper reference limit (mg/L)	HPLC reference interval (mg/L)	90% CI for HPLC upper reference limit (mg/L)
Boys	220	4-84	44-143	20-130[1]	87-194	2-117	70-311	16-239	147-443
Girls	220	5-145[2]	127-178	22-250[3]	214-424	4-367	218-472	16-576	284-642
Men	134	2-18	17-26	10-37	34-71	2-36	24-59	12-113	86-135
Women	146	2-34	25-50	14-62	51-79	2-43	33-47	8-107	91-136

[1]Two outliers were excluded prior to non-parametric estimation of the central 95%.

[2]Four outliers were excluded prior to non-parametric estimation of the central 95%.

[3]Two outliers were excluded prior to non-parametric estimation of the central 95%.

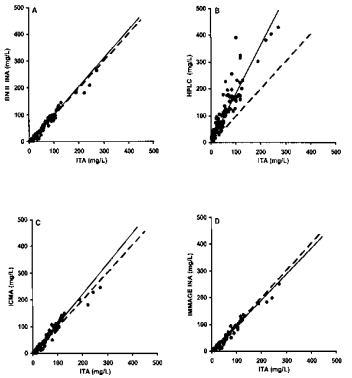

Fig. 1 Comparison of urinary albumin concentrations obtained by five methods using 100 patient samples. The solid line indicates Passing-Bablok regression line and the dashed line indicates x = y. In panel A, Passing-Bablok regression analysis gave a slope of 1.06 (1.00 to 1.09), an intercept of -4.8 (-5.9 to -2.0), and r = 0.983. In panel B, Passing-Bablok regression analysis gave a slope of 1.80 (1.66 to 2.02), an intercept of 2.4 (-2.0 to 10.4), and r = 0.912. In panel C, Passing-Bablok regression analysis gave a slope of 1.13 (1.08 to 1.18), an intercept of -5.0 (-6.4 to -3.4), and r = 0.976. In panel D, Passing-Bablok regression analysis gave a slope of 0.97 (0.94 to 1.00), an intercept of -3.9 (-5.0 to -1.8), and r = 0.984.

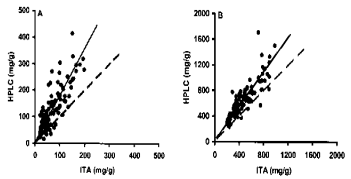

Fig. 2. HPLC versus ITA ACR method comparison. The solid line indicates Passing-Bablok regression line and the dashed line indicates x = y. In panel A, results from 101 samples with moderately elevated ACR (20-200 mg/g by ITA) are shown. Passing-Bablok regression analysis gave a slope of 1.77 (1.57-2.03), an intercept of 2.2 (-10.4-11.1), and r = 0.828. In panel B, results from 103 samples with markedly elevated ACR (200 to 1000 mg/g by ITA) are shown. Passing-Bablok regression analysis gave a slope of 1.33 (1.21-1.47), an intercept of 20.0 (-27.8-65.8), and r = 0.870.

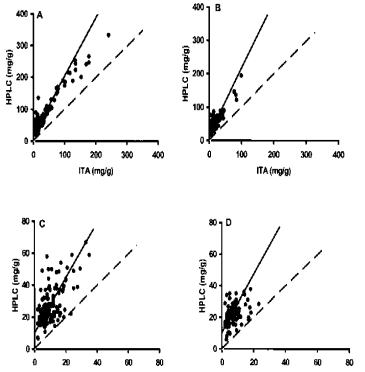

Fig. 3. Comparison of HPLC and ITA in normal subjects. The solid line indicates Passing-Bablok regression line and the dashed line indicates x = y. In panel A, results form 216 pediatric female samples are shown. Passing-Bablok regression analysis gave a slope of 1.78 (1.67-1.92), an intercept of 20.4 (18.4-22.6), and r = 0.956. In panel B, results from 216 pediatric male samples are shown. Passing-Bablok regression analysis gave a slope of 1.90 (1.70-2.14), an intercept of 18.4 (15.9-20.8), and r = 0.872. In panel C, results from 143 adult female samples are shown. Passing-Bablok regression analysis gave a slope of 1.73 (1.48-2.07), an intercept of 10.4 (7.4-12.1), and r = 0.625. In panel D, results from 132 adult male samples are shown. Passing-Bablok regression analysis gave a slope of 1.79 (1.47-2.21), an intercept of 10.7 (8.4-12.3), and r = 0.491.

Results and Discussion

The LOD was determined to be 3.4 mg/L, which is appropriate and comparable to the manufacturer's claim of 3 mg/L. The assay is was linear between 4.3 and 240 mg/L with a maximum deviation from a mean recovery of 100% at 66 mg/L. To verify that both HPLC and ITA assays were calibrated comparably, the linearity samples were also analyzed by ITA. Regression analysis of the HPLC versus ITA gave a slope of 0.91 with an intercept of 5.9 mg/L, indicating the two methods were calibrated comparably based on urine samples spiked with purified human serum albumin. Imprecision data are summarized in Table 1. Total CV's were less than 10% for all concentrations tested. The lowest concentration tested was 15.9 mg/L and it showed the highest CV. This indicates the assay has imprecision within published guidelines for MA testing. Passing-Bablok analysis comparing albumin concentrations measured by ITA, HPLC, two INA methods and ICMA for patient samples with moderately elevated albumin excretion rates are shown in (Fig. 1). There was a positive proportional bias for HPLC compared to all of the immunoassay methods while all four immunoassay methods showed good agreement. This suggests that immunoassay harmonization may have improved since earlier reports.[6,9] Passing-Bablok analysis comparing ACR by HPLC versus ITA for patient samples with moderately (Fig. 2A) and highly elevated (Fig. 2B) albumin excretion rates are shown. The overall correlations between ITA and HPLC (r = 0.83 and r = 0.87) were not as good as one might predict based on the relatively low imprecision of the two methods. HPLC shows a positive proportional bias compared to ITA for both patient types, but the disparity decreases when the amount of albuminuria increases. It has been proposed that for diabetic patients, immunoassay based methods considerably underestimate urinary albumin excretion, particularly for rates less than 100 µg/min. This has been attributed to the presence of an intact form of albumin that is immuno-unreactive but detectable by HPLC.[5] A summary of the non-parametric reference interval data for first or second morning void collections for adults and random collections for children is provided in the Table 2. The 90% confidence intervals for upper reference limits are also shown. These limits are generally quite broad even though there were more than 120 subjects in each group. It is noteworthy that 80% of boys, 83% of girls, 8% of men and 30% of women had an ACR by HPLC that was >30 mg albumin/g creatinine. When men and women were combined, 14% had an ACR by HPLC >30 mg/g. In addition to determining urinary microalbumin reference intervals for the HPLC and ITA methods in units of mg/g creatinine, we also determined reference intervals in units of mg/L (Table 2). It is noteworthy that when one compares adult male and female reference intervals using units of mg/L, the differences seen for the ACR disappear. Passing-Bablok analyses of comparisons between HPLC and ITA for the four reference groups are shown (Fig. 3). All groups show similar slopes indicating positive proportional bias for HPLC relative to ITA. The curves also illustrate the higher ranges for females vs. males for pediatric and adults subjects, and the approximately 2 to 1 ratio for HPLC vs. ITA at the upper reference limit. It is interesting that we see substantial amounts of immuno-unreactive albumin in our apparently healthy reference subjects. In adults the higher results by HPLC, which presumably correspond to immuno-unreactive albumin, might be partially attributable to undiagnosed underlying chronic conditions such as hypertension, type 2 diabetes mellitus, metabolic syndrome, and cardiovascular risk. However, in children this explanation seems less likely. The HPLC method has been reported to provide earlier detection of diabetic nephropathy by a mean of 3.9 years in type 1 diabetes mellitus and 2.4 years in type 2 diabetes mellitus compared with a radioimmunoassay method for urinary albumin measurement using timed urine samples.[7] Our data show that when using random urine samples from healthy subjects, the HPLC method yields albumin results that average nearly four times those of an ITA method and the upper reference limits for HPLC are double those of ITA. Further studies are needed with random urine samples to confirm that the HPLC method can indeed predict which patients will go on to develop diabetic nephropathy earlier than conventional immunoassay methods for urinary albumin. These studies should also determine method appropriate cutoff concentrations.

Conclusions

- The HPLC assay for urinary albumin studied shows good analytical performance.
- Further outcome studies are needed to evaluate how to best interpret urinary albumin results obtained by this HPLC method.

Acknowledgements

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