Performance Characteristics of an HPLC Assay for Urinary Albumin

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Abstract

Microalbuminuria is a marker of diabetic nephropathy and cardiovascular risk. Immunoassays underestimate the amount of intact albumin present in urine. The purpose of this study was to evaluate a new urinary albumin assay that uses size exclusion high-performance liquid chromatography (HPLC). We determined the limit of detection, linearity, imprecision, a comparison with an immunoturbidimetric assay, and pediatric and adult reference intervals. The limit of detection was 3.4 mg/L. The assay was linear from 4 to 240 mg/L. Total imprecision was less than 10% from 16 to 206 mg/L. Comparison of the albumin/creatinine ratio by HPLC with an immunoturbidimetric method showed positive proportional bias, which decreased with increasing concentrations of albumin. Nonparametric reference intervals were 22 to 250 mg/g for girls, 20 to 130 mg/g for boys, 14 to 62 mg/g for women, and 10 to 37 mg/g for men. This HPLC assay for urinary albumin shows acceptable performance and quantifies albumin species that are not detected by immunoassay. Separate reference intervals for children and adults seem necessary.
compare it with a conventional microalbumin immunoassay in healthy and diabetic subjects. The need for robust reference intervals for children and adults for this assay was a primary focus for the study.

Materials and Methods

The Accumin urinary albumin assay (AusAm Biotechnologies, New York, NY) uses size exclusion HPLC with UV detection. A Zorbax Bio Series GF-250 column was provided with the kit. The assay was performed on an Agilent 1100/HP (Agilent Technologies, Los Angeles, CA) instrument according to the manufacturer’s instructions. The chromatographic peak corresponding to intact albumin has been shown to be free of contamination by other urinary proteins.12

The Wako Microalbumin B comparison method (Wako Diagnostics, Richmond, VA) is an automated immunoturbidimetric assay (ITA) that uses monoclonal and polyclonal antibodies to human albumin. The amount of turbidity produced by albumin-antibody complexes is measured optically and is directly proportional to the albumin concentration. The assay was run on a Roche MODULAR P analyzer (Roche Diagnostics, Indianapolis, IN).

Two immunonephelometric assays for microalbumin also were used. One was performed on the BN II nephelometer using the manufacturer’s reagents (Dade Behring, Deerfield, IL). The second was performed on an IMMAGE nephelometer using the manufacturer’s reagents (Beckman Coulter, Brea, CA). An immunochemiluminometric assay for microalbumin was performed on an IMMULITE 2000 analyzer using reagents from the manufacturer (Diagnostic Products, Los Angeles, CA).

Urine creatinine was quantified by the Jaffe method on a Roche MODULAR P analyzer using Roche reagents. The Wako ITA and Roche creatinine assays were used routinely in our clinical laboratory at the time of this study, and both were performed according to the manufacturers’ instructions.

The limit of detection 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 with the average amplitude of the low calibrator, which had a concentration of 9.41 mg/L. From the relationship of amplitude to albumin, a concentration of albumin equivalent to 5 times the background noise was determined to be the limit of detection.

Linearity studies were performed using dilute urine from a healthy male volunteer (low pool) and supplementing it 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 3 concentrations of albumin. Each imprecision pool was divided into aliquots and frozen at –70°C until it was analyzed. Sufficient aliquots were prepared to perform 2 runs in duplicate per day for 10 days, for a total of 40 replicates per pool.

Method comparison samples with moderate albumin/creatinine ratios (ACRs), defined as 20 to 200 mg/g (n = 101), and high ACR samples defined as 200 to 1,000 mg/g (n = 103) were retrieved from our patient sample frozen storage at –20°C. Many of these samples that were submitted for routine testing were presumably from patients with diabetes mellitus. They then were deidentified and placed at –70°C until testing. Before analyzing for albumin by HPLC, the samples were thawed, gently mixed by vortexing, and centrifuged at 3,000g for 10 minutes. This albumin result was used to calculate the ACR using the original creatinine result.

Adult reference samples were obtained 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 during the last month, and women who were menstruating.13 Our reference subjects included 80 men and 80 women ranging in age from 19 to 66 years. The samples were first or second morning voided samples.14 Aliquots were frozen at –70°C until analysis. Because our reference study subjects live at a moderate altitude (1,370 m), we arranged for reference samples to be obtained from healthy volunteers living at sea level to account for any effect of altitude.15-17 The sea level samples were shipped frozen to our facility from their collection point. These samples were chiefly from young adults (66 women and 54 men with an age range from 18 to 26 years except for 1 subject who was 46 years old). The samples were stored at –70°C until testing. Pediatric reference samples were random collections from healthy children who were taking no prescription medications and included 20 boys and 20 girls for each year of life from 7 to 17 years, for a total of 440 samples.

All reference samples were handled preanalytically as described earlier. They were analyzed for albumin (ITA) and creatinine using the MODULAR P assays and then by the HPLC assay. 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).

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, Kennett Square, PA) was used for evaluation of linearity, imprecision, Passing-Bablok regression, calculation of r, and reference interval estimation.
Results

The limit of detection of the assay based on a signal/noise ratio of 5 was 3.4 mg/L. Linearity of the HPLC assay was tested in a range of albumin concentrations from 4.3 to 240 mg/L. The maximum deviation from a mean recovery of 100% was 4.6% at a concentration of 66 mg/L. To verify that the HPLC and ITA assays were calibrated comparably, the linearity samples also were analyzed by ITA. Regression analysis of HPLC vs ITA yielded a slope of 0.91 and an intercept of 5.9 mg/L, indicating the methods were calibrated comparably based on urine samples spiked with purified human serum albumin. Imprecision data are summarized in Table 1. Total coefficients of variation were less than 10% for all 3 concentrations of albumin tested. The lowest concentration tested was 15.9 mg/L, and it showed the largest coefficient of variation.

Passing-Bablok analysis comparing albumin concentrations measured by ITA, HPLC, 2 immunonephelometric assay methods, and an immunochemiluminometric assay for patient samples with moderately elevated albumin excretion rates are shown Figure 1. The immunoassay methods gave comparable results, but the HPLC method gave albumin concentrations that generally were substantially higher than those from the ITA. The results of Passing-Bablok analyses comparing ACR by HPLC vs ITA for patient samples with moderately Figure 2A and highly elevated Figure 2B albumin excretion rates are shown. The overall correlations between ITA and HPLC (r = 0.828 and r = 0.870) were not as good as one might predict based on the relatively low imprecision of the 2 methods. The slopes for the 2 groups of subjects are significantly different (P < .01). The results indicate a positive proportional bias for the HPLC method relative to the conventional ITA method, which decreases with increasing albumin excretion rates.

Results from pediatric and adult reference samples were evaluated for differences between males and females in ACRs. For the HPLC method, significant differences were found between males and females for both age groups. When samples from men obtained at sea level were compared with those collected at 1,370 m above sea level, a small but statistically significant difference was detected; however, when samples for similarly aged subjects were compared (19-26 years), no significant difference could be demonstrated (P = .57). This same age relationship was seen for women. This indicated no relationship between altitude and ACR determined by HPLC.

Table 1

<table>
<thead>
<tr>
<th>Pool Mean (mg/L)</th>
<th>Within-Run CV (%)</th>
<th>Between-Day CV (%)</th>
<th>Total CV (%)</th>
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<tbody>
<tr>
<td>15.9</td>
<td>4.9</td>
<td>5.7</td>
<td>8.4</td>
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<td>89.3</td>
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<tr>
<td>205.7</td>
<td>0.7</td>
<td>2.7</td>
<td>2.9</td>
</tr>
</tbody>
</table>

CV, coefficient of variation.

Figure 1

Comparison of urinary albumin concentrations obtained by 5 methods using 100 patient samples. The solid line indicates the Passing-Bablok regression line, and the dashed line indicates x = y. Slope and intercept statistics for Passing-Bablok analysis are given as the median (95% confidence interval in parentheses). A, Passing-Bablok regression analysis gave a slope of 1.06 (1.00-1.09), an intercept of –4.8 (–5.9 to –2.0), and r = 0.983. B, Passing-Bablok regression analysis gave a slope of 1.80 (1.66-2.02), an intercept of 2.4 (–2.0 to 10.4), and r = 0.912. C, Passing-Bablok regression analysis gave a slope of 1.13 (1.08-1.18), an intercept of –5.0 (–6.4 to –3.4), and r = 0.976. D, Passing-Bablok regression analysis gave a slope of 0.97 (0.94-1.00), an intercept of –3.9 (–5.0 to –1.8), and r = 0.984. HPLC, high-performance liquid chromatography; ICMA, immunochemiluminometric assay; INA, immunonephelometric assay; ITA, immunoturbidimetric assay.
ACR results for all adult male reference subjects were combined and assessed for outliers, which were excluded from further analysis. All adult female ACR results were evaluated similarly. Pediatric male and female samples were correlated against age and examined for the presence of outliers. No significant correlation was seen with age for pediatric samples from boys or girls.

ACR by ITA for these reference samples was evaluated by the same statistical methods. The only significant difference seen was for males and females, similar to HPLC results.

Men and women were partitioned in 2 age groups, young adult (18-26 years) and adult (27-60+ years). HPLC and ITA showed no significant difference (Z score < Z critical). Analysis of results for boys and girls compared with those for men and women showed that the groups were significantly different (Z score > Z critical) and should be partitioned.

A summary of the nonparametric reference interval data for first or second morning voided samples for adults and random collections for children is provided in Table 2. The 90% confidence intervals for the upper reference limits also are shown. These limits generally are quite broad, even though there were more than 120 subjects in each group. It is noteworthy that 80.3% of boys, 83.0% of girls, 8.2% of men, and 29.5% of women had an ACR by HPLC that was more than 30 mg of albumin per gram of creatinine. When samples from men and women were combined into 1 group, 19.3% had an ACR by HPLC of more than 30 mg/g. In addition to determining urinary microalbumin reference intervals for the HPLC and ITA methods in milligrams per gram of creatinine, we also determined reference intervals in milligrams per liter (Table 2). It is noteworthy that when one compares reference intervals for men and women using milligrams per liter, the differences seen for the ACR disappear.

Passing-Bablok analyses of comparisons between HPLC and ITA for the 4 reference groups are shown. All groups showed 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 adult subjects and the approximately 2:1 ratio for HPLC vs ITA at the upper reference limit.

**Discussion**

The HPLC assay for urinary albumin had an appropriately low limit of detection (3.4 mg/L) that was comparable to the manufacturer’s claim of 3 mg/L. The assay was linear across
an appropriate albumin concentration range. It demonstrated imprecision that was well within published guidelines at all albumin concentrations tested.14

There was a positive proportional bias for HPLC compared with all immunoassay methods used to quantify the albumin concentration (Figure 1). It is noteworthy that all 4 immunoassay methods showed good agreement. This suggests that immunoassay harmonization might have improved since earlier reports.6,9 There also was a proportional bias for HPLC compared with ITA for patients with elevated ACRs (Figure 2). This disparity, however, decreased when the amount of albuminuria increased.

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 not immunoreactive but is detectable by HPLC.8 Albumin that is not immunoreactive seems to have a limited number of polypeptide chain scissions and is held together by noncovalent intrachain bonding disulfide bonds.12 It is interesting that we found substantial amounts of this nonimmunoreactive albumin in our apparently healthy reference subjects. The slopes of HPLC vs ITA (Figure 3) and a comparison of the HPLC and ITA upper reference limits (Table 2) confirm similar ratios of total and immunoreactive albumin, as seen in patients with moderate increases in ACRs. Although the ratio of microalbumin measured by HPLC to that measured by ITA averages nearly 4, data are scattered considerably about the regression line. In healthy subjects, this ratio can be as low as 1 and as high as 16.

There was no significant difference between results for adult reference samples obtained at sea level and at 1,370 m from subjects of comparable age. This finding differs from previous reports.15-17 However, those studies were done at much higher altitudes and involved acute exposure rather than long-term acclimatization such as our reference subjects who live at a moderate altitude have experienced. We saw no significant difference between younger and older adults, but our study included only 3 subjects older than 60 years, with a maximum age of 66 years. It has been shown that the ACR does not begin to rise significantly in apparently healthy adults until the 60- to 80-year range.13,21 The 97.5 percentile values for the ITA assay of 34 and 18 mg/g we obtained are comparable with published cutoffs of 30.9 and 22.1 mg/g for females and males, respectively.22 The higher ACR reference limits for females compared with males might be due to higher urine creatinine concentrations in males. It has been suggested that the absolute concentration of urinary albumin can be used clinically.23 When one compares albumin concentrations quantified by HPLC or ITA, the differences between men and women are reduced markedly.

Our results for pediatric reference subjects indicate a disparity in the ACR between boys and girls similar to that seen in adults. However, this disparity also was seen for the albumin concentration not corrected for creatinine. Furthermore, the ACR and albumin concentrations for children were higher than for adults. This may be due in part to the fact that most pediatric samples were not first morning specimens. Random urine specimens collected throughout the day might be more dilute, and albumin excretion might be increased owing to...
physical activity. We observed a positive correlation between pediatric urine creatinine concentrations and age; however, we saw no such correlation between age and the ACR for the HPLC or ITA methods (data not shown).

In contrast with our findings, a phenomenon of increasing albumin excretion has been shown in children with type 1 diabetes mellitus, particularly during puberty, when timed urine collections were evaluated. The pediatric 97.5 percentile reference limits for ACR by HPLC were twice those for the ACR by ITA, which was similar to what was seen for adults. In adults, the higher results by HPLC, which presumably correspond to nonimmunoreactive albumin, might be attributable partially 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. It seems that in apparently healthy individuals, the concentration of albumin quantifiable in the urine by HPLC averages nearly 4 times the concentration quantifiable by ITA. This difference between methods does not seem due to calibration differences because samples spiked with human serum albumin gave comparable results.

It is noteworthy that the 90% confidence intervals for the upper reference limits were quite large, even though all subject groups included more than 120 individuals. This is due in part to the highly skewed distribution of reference values that were encountered with both methods used to measure urinary albumin. For boys, the upper reference limits for ITA and HPLC overlap for the ACR and albumin concentration. For girls, the upper reference limits for ITA and HPLC overlap only for the albumin concentration, indicating that separate reference intervals for ITA and HPLC are necessary. For adults, there is no overlap in the upper reference limits for ITA and HPLC, and separate reference intervals clearly are needed.

The 90% confidence intervals for males and females for the ACR reveal overlap for boys and girls by ITA but not HPLC, indicating the need for separate reference intervals for the ACR by HPLC in boys and girls. For men and women, the 90% confidence intervals for the ACR for the ITA and HPLC assays overlap, suggesting that the same reference intervals can be used for men and women. These single reference intervals are 2 to 26 mg/g of creatinine for ITA (90% confidence interval for upper reference limit, 21-35 mg/g) and 10 to 58 mg/g of creatinine for HPLC (90% confidence interval for upper reference limit, 50-67 mg/g). A larger study in adults is needed to confirm that separate reference intervals for men and women are unnecessary.

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. Our data show that when using random urine samples from healthy subjects, the HPLC method yields albumin results that average nearly 4 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 predict earlier than conventional immunoassay methods for urinary albumin which patients will develop diabetic nephropathy. These studies also should determine method-appropriate cutoff concentrations. For current immunoassay methods for urinary albumin, the clinical decision cutoff for diabetic nephropathy is higher than the upper reference limit. A recent editorial called for new reference intervals for the HPLC method. However, what is needed for diabetic nephropathy is an appropriate clinical decision cutoff, which could be the same or even lower than the upper reference limit. Given the nearly 2-fold difference in the upper reference limit for the ACR in men and women by ITA of 18 and 34 mg/g, respectively, a single adult cutoff might not be optimal for conventional urine albumin immunoassays. Additional studies examining the relationship between urinary albumin measured by the HPLC method and cardiovascular risk also are needed. It might be that some of our apparently healthy reference subjects actually are at increased risk for cardiovascular disease.

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