Measurement of Microalbuminuria Using Protein Chip Electrophoresis

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Key Words: Microalbuminuria; Chip; Electrophoresis; Protein analysis; Microfluidics; Immunounreactive albumin

Abstract

Microalbuminuria reflects the progression of nephropathy and cardiovascular disease in diabetic and hypertensive patients. Most commercially available tests currently used to measure microalbuminuria are immunoassays. We developed a microfluidics-based assay using the P200 protein chip (Caliper Life Sciences, Mountain View, CA, and Agilent Technologies, Santa Clara, CA) and 2100 Bioanalyzer (Agilent Technologies) to detect microalbuminuria. The method integrates and automates the electrophoretic separation and fluorescent detection of proteins from 14 to 200 kd. The assay was linear up to 750 mg/L and demonstrated good sensitivity with a lower detection limit of 7.5 mg/L. Intrachip and interchip coefficients of variation ranged from 0% to 4% and 4.9% to 13.5%, respectively. When albumin was measured by chip and immunoturbidimetry in diabetic urine samples, the chip consistently showed higher albumin concentrations. The discrepancy may be due to the chip’s ability to detect immunounreactive albumin. Overall, this simple, cost-effective assay offers a sensitive and accurate measurement of microalbuminuria that can be easily implemented in a clinical laboratory.

Fourteen million people in the United States have diabetes mellitus, and diabetic nephropathy is, not surprisingly, the country’s leading cause of end-stage renal disease.1,2 Microalbuminuria is considered a marker of incipient nephropathy in people with diabetes, and, thus, screening for microalbuminuria is performed routinely in people with type 1 and type 2 diabetes.3-5 Microalbuminuria refers to urinary albumin excretion that is more than the normal range but less than levels that can be detected by a urine dipstick.6 Microalbuminuria is defined as 30 to 300 mg of albumin in a 24-hour urine sample or an albumin/creatinine ratio (ACR) of 0.03 to 0.3 in a random urine sample, preferably the first morning void.3,7 If microalbuminuria is detected early in patients with diabetes, the onset of nephropathy and cardiovascular disease can be prevented with drugs that inhibit the renal angiotensin-aldosterone system.8-13

It is interesting that prospective studies have demonstrated that microalbuminuria can predict cardiovascular disease and premature mortality in hypertensive patients and even in the general population.6,12,13 It is postulated that microalbuminuria is associated with nephropathy and cardiovascular disease because it is an indicator of generalized vascular dysfunction. In addition, microalbuminuria itself may damage the kidney by inducing production of inflammatory agents.12

Most commercially available tests to quantify urinary albumin are immunoassays, such as immunoturbidimetry, immunonephelometry, and radioimmunoassay.14 The immunochromical methods are sensitive, but recent studies suggest that after glomerular filtration, some albumin may be processed by lysosomes to create an immunounreactive form of intact albumin that cannot be detected by immunoassays.15 High-performance liquid chromatography (HPLC) and gel
Materials and Methods

Equipment

The Caliper Protein 200 Chip used with the Agilent 2100 Bioanalyzer is a chip-based electrophoretic assay that can separate and detect proteins from 14 to 200 kd. The Agilent 2100 Bioanalyzer, the Protein 200 LabChip kit, and protein chips are distributed by Agilent Technologies. A chip priming station and chip assay software are supplied with the Bioanalyzer. Each chip measures 2.0 × 2.0 × 0.3 in (5.0 × 5.0 × 0.8 cm), and the 2100 Bioanalyzer, which weighs 22 lb (9.9 kg) and measures 6.4 × 16.2 × 11.4 in (16.3 × 41.1 × 29.0 cm), fits easily on a desktop or laboratory bench. The software integrates and automates the steps of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). We tested the assay for linearity, precision, and accuracy. Owing to the impracticality of doing HPLC and gel electrophoresis in most clinical laboratories and the questionable accuracy of HPLC, we focused our investigation on comparing the chip assay with a commonly used immunoturbidimetric method for quantifying albumin in urine samples from patients with diabetes.

Methods

This protein assay, performed on a small chip in conjunction with the Agilent 2100 Bioanalyzer and accompanying software, integrates and automates multiple electrophoretic steps (sample handling, separation, staining, destaining, and detection) and combines it with digital data processing. Each chip has 2 glass layers bonded together, one of which contains photolithographically etched microchannels. There are 16 wells on a chip, 10 for samples.

Before loading a sample onto the chip, the chip’s channels were filled with the prepared gel-dye mixture (which contains SDS, dye, and sieving matrix) by pipetting 12 µL of gel-dye mix into one of the wells and applying pressure for 1 minute with a syringe in the chip priming station. The gel-dye mix was also loaded into 3 other wells, destaining solution into 1 well, and sizing ladder into 1 well. For sample preparation, 30 µL of the protein sample was added to 15 µL of the prepared denaturing solution. The sample was heat denatured for 4 minutes in boiling water and then diluted with 630 µL of purified water. (Different volumes of sample, denaturing solution, and water can be used, but the same ratios need to be maintained.)

Up to 10 samples can be loaded onto 1 chip (6 µL per well), and, after sample loading, the chip is placed in the Agilent 2100 Bioanalyzer. The bioanalyzer contains electrodes
that touch the samples in each well, creating an electric circuit, which then moves the samples from the wells into the channels. The SDS in the channels surrounds protein in the sample forming SDS-protein micelles that interact with the fluorescent dye. Each sample is then sequentially injected into the separation channel in the center of the chip where size-based protein separation and detection by a laser fluorescent detection system (630 nm) occur.²¹,²²

Analysis of 10 samples takes 25 to 30 minutes, and the results are displayed on a computer screen in a gel-like image, as an electrophoretogram, or in tabular formats, with each protein at its specific separation time based on size and corresponding fluorescence level reflecting protein concentration. Chicken albumin, human albumin, and β-galactosidase separate out at approximately 26, 28, and 33 seconds, respectively.

Samples containing purified human albumin at different dilutions were used for linearity, calibration, and precision analysis. Urine samples from 48 patients with diabetes and urinary albumin levels ranging from 4.0 to 432.8 mg/L by immunoturbidimetry were run on the chip assay, and results were compared with the immunoturbidimetry measurements. The urine creatinine level for each sample was also measured to determine and compare the ACR by each method.

Results

Linearity

Linearity was assessed by using sequential dilutions of the stock human albumin standard. The electrophoretograms obtained from 10 concentrations of human albumin loaded on a single chip are shown in Figure 1I. The ratio of the human albumin peak area to the chicken albumin peak area (referred to as relative units) was obtained for known human albumin concentrations (7.5, 15, 30, 60, 75, 112.5, 150, 225, 300, 375, and 750 mg/L) with the chip assay. The relative units were then plotted against the known concentrations of human albumin in each sample. We found that for relative units greater than 3.0 (equals 150 mg/L of human albumin), the generated curve was linear Figure 2A; and the equation \( y = 50.6x \) was used to calculate absolute albumin concentrations in urine samples yielding relative units more than 3.0. For relative units less than 3.0, the data points were nearly linear but were best fit with a polynomial function, which was used to calculate the absolute albumin concentration in urine samples yielding relative units less than 3.0 Figure 2B.

Precision

Evaluation of precision was performed with low, medium, and high concentrations of human albumin. For intrachip precision assessment, 30, 112.5, and 375 mg/L of human albumin were run in 5 sample wells of 1 chip. Also, 75 mg/L of human albumin was run in all 10 wells of 5 different chips. The intrachip coefficients of variation for the 4 albumin concentrations were 0.0% to 4.0% Table 1. For interchip precision, concentrations ranging from 7.5 to 375 mg/L were run on 6 chips with coefficients of variation between 4.9% and 13.5% Table 2. Finally, day-to-day precision was also assessed and results are shown in Table 3.

Comparison Studies

Albumin measurements in urine samples obtained with the P200 chip were compared with albumin concentrations obtained using a conventional immunoassay (immunoturbidimetry). The 48 urine samples were from patients with diabetes and had concentrations of albumin ranging from 4.0 to 432.8 mg/L as measured by immunoturbidimetry. Figure 4 shows an example of an electrophoretogram from one of the patient samples, which was created by the chip’s data analysis software. A graph comparing albumin concentrations detected by immunoturbidimetry vs chip electrophoresis for each sample is shown in Figure 4I, and the best-fit line yields a correlation of \( R^2 = 0.917 \).

The mean albumin concentrations measured by immunoturbidimetry and by chip electrophoresis were 98.2 and 167.5 mg/L, respectively; and for all the urine samples tested, the albumin concentration measured by the chip was greater.
than that measured by immunoturbidimetry. Because random urine samples were used, the urinary creatinine level for each sample was measured to calculate the ACR. With a definition of microalbuminuria as an ACR between 0.03 and 0.3, 19 samples were normoalbuminuric, 24 were microalbuminuric, and 5 were macroalbuminuric by immunoturbidimetry. For the 19 samples that were classified as normoalbuminuric, the mean ± SD albumin concentration was 19.9 ± 16 mg/L using immunoturbidimetry vs 53.8 ± 28.8 mg/L using the P200 Chip. Keeping the definition of microalbuminuria as an ACR between 0.03 and 0.3, 14 of 19 samples that were normoalbuminuric by immunoassay were actually microalbuminuric by chip electrophoresis. For the 24 samples that were microalbuminuric by immunoturbidimetry, the mean ± SD albumin concentration was 97.0 ± 72.8 mg/L for the immunoassay vs 180.6 ± 126.7 mg/L for chip electrophoresis. Although for these 24 samples the absolute albumin measurement by the chip method was greater than by the immunoassay, they were

Table 1

<table>
<thead>
<tr>
<th>HA (mg/L)</th>
<th>Mean Relative Units</th>
<th>SD (mg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.34</td>
<td>0.013</td>
<td>4.0</td>
</tr>
<tr>
<td>75</td>
<td>0.97-1.0</td>
<td>0.007-0.023</td>
<td>0.07-1.3</td>
</tr>
<tr>
<td>112.5</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>375</td>
<td>7.52</td>
<td>0.15</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* The human albumin (HA) concentrations represent dilutions of stock human albumin. For 30, 112.5, and 375 mg/L, the samples were run in 5 wells of a single chip and the mean of the 5 wells for each concentration was calculated (mean relative units). The SD and coefficient of variation (CV) were determined from the mean. In addition, 75 mg/L was run in all 10 wells of 5 chips, and the ranges in the mean, SD, and CV for the 5 chips are shown.

Table 2

<table>
<thead>
<tr>
<th>HA (mg/L)</th>
<th>Mean Relative Units</th>
<th>SD (mg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>0.037</td>
<td>0.005</td>
<td>13.5</td>
</tr>
<tr>
<td>15</td>
<td>0.095</td>
<td>0.009</td>
<td>9.5</td>
</tr>
<tr>
<td>30</td>
<td>0.32</td>
<td>0.024</td>
<td>7.5</td>
</tr>
<tr>
<td>60</td>
<td>0.617</td>
<td>0.042</td>
<td>6.8</td>
</tr>
<tr>
<td>75</td>
<td>1.028</td>
<td>0.05</td>
<td>4.9</td>
</tr>
<tr>
<td>112.5</td>
<td>1.883</td>
<td>0.15</td>
<td>8.0</td>
</tr>
<tr>
<td>150</td>
<td>3.033</td>
<td>0.24</td>
<td>8.0</td>
</tr>
<tr>
<td>225</td>
<td>4.717</td>
<td>0.43</td>
<td>9.1</td>
</tr>
<tr>
<td>300</td>
<td>5.967</td>
<td>0.56</td>
<td>9.4</td>
</tr>
<tr>
<td>375</td>
<td>7.367</td>
<td>0.75</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* Ten known concentrations of human albumin (HA) were run in a single well of 6 chips. For each concentration, the mean and SD over the 6 chips were calculated. The coefficients of variation (CVs) ranged from 4.9% to 13.5%.

Table 3

<table>
<thead>
<tr>
<th>HA (mg/L)</th>
<th>Mean Relative Units</th>
<th>SD (mg/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.38</td>
<td>0.02</td>
<td>5.3</td>
</tr>
<tr>
<td>75</td>
<td>0.98</td>
<td>0.03</td>
<td>3.6</td>
</tr>
<tr>
<td>225</td>
<td>4.60</td>
<td>0.14</td>
<td>3.1</td>
</tr>
<tr>
<td>375</td>
<td>7.35</td>
<td>0.07</td>
<td>0.96</td>
</tr>
</tbody>
</table>

CV, coefficient of variation; HA, human albumin.

* Each concentration was run in a single well of a chip on 2 separate days yielding CVs between 0.96% and 5.3%.
all still in the microalbuminuric range when the ACR was calculated. For the 5 samples with an ACR of more than 0.3 by immunoturbidimetry (macroalbuminuria), the mean ± SD albumin concentration was 401.6 ± 31.0 mg/L by immunoturbidimetry and 536.4 ± 131.1 mg/L by chip electrophoresis. These comparisons are summarized in Table 4.

### Discussion

The P200 chip assay was proven to be a sensitive and precise method for measuring urinary albumin in this study. With a lower limit of detection of 7.5 mg/L, it offers a good alternative to the currently used immunoassays. Furthermore, we found that the protein chip consistently demonstrated a higher amount of urinary albumin with diabetic urine samples than did immunoturbidimetry. Similar findings were also seen with native PAGE and with HPLC.

A similar discrepancy was also seen in our study in that 14 patient samples identified as normoalbuminuric by immunoturbidimetry measurements were bumped into the microalbuminuric category with the chip’s albumin measurement. However, none of the samples identified as microalbuminuric by immunoturbidimetry were bumped up into the macroalbuminuric category by the chip method.

The higher concentration of urinary albumin detected by nonimmunochemical methods may be due to their ability to measure modified albumin. After glomerular filtration, albumin is modified by lysosomal enzymes, creating some albumin molecules that are intact but no longer immunoreactive owing to “nicking.” Grieve et al.15 demonstrated that streptozotocin-induced diabetic rats and control rats contained

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**Table 4** Chip vs Immunoturbidimetry for Determining Microalbuminuria in 48 Urine Samples

<table>
<thead>
<tr>
<th></th>
<th>Immunoturbidimetry</th>
<th>Chip Electrophoresis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. of Samples</td>
<td>Albumin Concentration (mg/L)</td>
</tr>
<tr>
<td>Normoalbuminuria (ACR &lt;0.03)</td>
<td>19</td>
<td>19.9 ± 16</td>
</tr>
<tr>
<td>Microalbuminuria (ACR, 0.03-0.3)</td>
<td>24</td>
<td>97.0 ± 72.8</td>
</tr>
<tr>
<td>Macroalbuminuria (ACR &gt;0.3)</td>
<td>5</td>
<td>401.6 ± 31.0</td>
</tr>
</tbody>
</table>

ACR, albumin/creatinine ratio.

* For better comparison of results, the urine samples were divided into 3 groups (normoalbuminuria, microalbuminuria, and macroalbuminuria) based on the immunoturbidimetric albumin measurement and the ACR definition of microalbuminuria. The number of samples classified as normoalbuminuric, microalbuminuric, or macroalbuminuric by immunoturbidimetry and by chip electrophoresis are shown for each group, and the mean ± SD in each group. As shown, more patients are classified as having microalbuminuria when the chip measurement is used. Albumin concentrations are given as mean ± SD.
intact albumin in the urine that could not be detected by immunochemical methods. They found that the immunonunreactive albumin excretion rate increased 11-fold after 8 weeks of streptozotocin-induced diabetes as compared with that in control rats. This immunonunchemically nonreactive form of albumin has also been isolated and purified in urine samples from patients with diabetes.19

Thus, the renal modification of albumin causes the excretion of intact immunonunreactive albumin, which can then be detected only by nonimmunochemical methods. It was once thought that HPLC was the most specific test to measure immunonunreactive and immunonunreactive forms of intact urinary albumin; however, the accuracy and specificity of size-exclusion HPLC has come into question. Sviridov et al20 reported that urinary albumin measurements by size-exclusion HPLC may overestimate the amount of urinary albumin owing to several globulins that elute at the same time as albumin, such as transferrin, α₁-antitrypsin and α₁-acid glycoprotein, all of which can also be present in urine. Thus, it seems that HPLC does not specifically detect albumin in urine because its albumin measurement may be including not only intact albumin (immunonunreactive and immunonunreactive), but also several other urinary globulins. Furthermore, HPLC and conventional PAGE are not practical in most clinical laboratories. HPLC is costly and difficult to perform, and PAGE is labor-intensive and time-consuming. Thus, there is a need to develop a nonimmunologic method that can be easily used in a clinical laboratory setting and detects all forms of intact urinary albumin with good sensitivity and specificity.

As mentioned, the protein chip is sensitive and can detect albumin concentrations as low as 7.5 mg/L. Also, like the other nonimmunochemical techniques, the chip consistently demonstrated a higher albumin concentration than did immunoturbidimetry for all samples tested. Thus, this assay, using a technique equivalent to reducing SDS-PAGE, is likely detecting immunonunreactive forms of albumin causing the apparently increased albumin measurements. Although one study showed that reducing-SDS conditions caused a major loss of immunonunchemically unreactive albumin, we postulate that the reducing conditions used in our study were not as strong, and, thus, some immunonunreactive intact albumin remained to be measured.

With electrophoretic methods, other urinary proteins do not seem to be separating out at the same band position as urinary albumin. In a study by Osicka and Comper,3 diabetic urine samples were run by native PAGE, and analysis of the band on the gel that migrated the same distance as the albumin calibrator was shown to contain only albumin by liquid chromatography/mass spectrometry/mass spectrometry. Furthermore, one study that implemented a protein chip technique similar to the method used in the present study demonstrated that purified samples of transferin, α₁-antitrypsin, and α₁-acid glycoprotein were resolved separately and clearly from the albumin peak.23

Because the traditionally used immunoassays apparently underestimate the amount of intact albumin excreted into urine, methods that can also detect immunonunreactive forms of intact albumin may bear some clinical significance. It has been shown that in patients with type 1 and type 2 diabetes, measurement of albumin by HPLC can predict the onset of persistent albuminuria 3.9 and 2.4 years earlier, respectively, than measurement of urinary albumin by radioimmunoassay.24 In the general population, HPLC measurements correlated better with the presence of peripheral vascular disease and diabetes, although immunoassay measurements correlated better with cardiovascular risk factors. An editorial noted that HPLC is not a readily usable technique in most clinical laboratories and suggested the use of electrophoretic methods to assay urinary proteins.25 The P200 protein chip may provide such a method in that it is a practical assay that provides a sensitive measurement of microalbuminuria that, in reality, may be more specific and accurate than HPLC. However, more work is needed to establish reference intervals for the chip-based methods and to determine if the values obtained by on-chip electrophoresis correlate with clinically significant outcomes.

References


