Chip Electrophoresis as a Method for Quantifying Total Microalbuminuria

Owen T.M. Chan 1,2 and David A. Herold 1,2*

**Background:** Microalbuminuria is an important prognostic marker in diabetic nephropathy and cardiovascular disease. Initially, most commercial assays used immunoreactivity to quantify microalbuminuria; however, size-exclusion HPLC demonstrated the existence of non-immunoreactive forms of albumin that may not be detected by immunoassay. Recent liquid chromatography tandem mass spectrometry analyses suggested that size-exclusion HPLC gave higher results attributable to other urine proteins coeluting with albumin. We describe an assay that measures total microalbuminuria (immunoreactive and nonimmunoreactive) without any discernable interference from other common urine proteins.

**Methods:** We used an automated chip electrophoresis system that utilized microfluidic separation technology and fluorescent sample detection. Each albumin specimen was mixed with the manufacturer's sample buffer in addition to a chicken albumin internal calibrator and then electrophoresed without additional reducing agents.

**Results:** With variable concentrations of bovine serum albumin normalized to a chicken albumin internal calibrator, the electrophoresis system was best fit with a polynomial ($R^2 = 0.9997$; concentration range, 5–300 mg/L). The lower limit of detection was 5 mg/L. Interchip and intrachip variation studies conducted on patient urine demonstrated CVs of 3%–13%. The introduction of potentially interfering agents (i.e., molecular analytes, nonalbumin proteins) did not alter precision. Compared with immunoassay, the chip electrophoresis identified higher microalbuminuria concentrations in all urine samples. The method also clearly resolved the albumin peak from interfering proteins.

**Conclusions:** Unlike immunoassay, chip electrophoresis can detect both immunoreactive and nonimmunoreactive forms of albumin. This system is a simple, robust method to quantify microalbuminuria with good sensitivity, precision, and accuracy.

© 2006 American Association for Clinical Chemistry

The detection of microalbuminuria, defined as 30–299 mg albuminuria/24 h (20–199 μg/min) or 30–299 μg albumin/mg creatinine (1) has prognostic value for the general population. Microalbuminuria is an established risk factor of diabetic nephropathy and cardiovascular disease (2–11) and has a prevalence of 24.9% 10 years after diagnosis in patients with diabetes mellitus type 2 (12). Microalbuminuria is predictive of clinical proteinuria and early mortality for those with diabetes (5), and the PREVEND (Prevention of Renal and Vascular End stage Disease) study demonstrated that microalbuminuria is independently associated with increased cardiovascular risk factors and cardiovascular morbidity in nondiabetic, nonhypertensive persons (3). Ongoing studies are investigating whether microalbuminuria would be a good target for drug therapy and subsequent protection against renal disease and cardiovascular events (13, 14).

Given the clear association of microalbuminuria with injury, the accurate detection of urinary albumin is critical in detecting disease and monitoring its progression. With current assays, however, the presence of nonimmunoreactive urinary albumin frequently leads to an underestimation of microalbuminuria (15–17). The accuracy of HPLC, used to identify both reactive and nonimmunoreactive urinary albumins (17–20), was questioned because several urinary globulins (i.e., α1-antitrypsin, transferrin) coeluted with albumin when size-exclusion HPLC was used to quantify albuminuria (21).

We developed an assay that uses chip electrophoresis (22) to quantify microalbuminuria. To test the assay performance, we analyzed a variety of substances found in patient urine to see whether they would alter the assay’s precision and function, and we tested urinary,
nonalbumin proteins to see if they coeluted with albumin and thus interfered with quantification. We also compared detection of patient microalbuminuria by conventional immunoassay with detection by our method.

**Materials and Methods**

**CHIP ELECTROPHORESIS**

We adapted the Experion™ Pro260 Analysis Kit (Bio-Rad Laboratories) for the quantitative determination of albumin in urine by adding chicken albumin (CA)³ as an internal calibrator. The Experion automated electrophoresis system utilizes LabChip microfluidic separation technology and fluorescent sample detection (Caliper Life Sciences) to perform automated analysis of 10 protein samples per chip. Briefly, sodium dodecyl sulfate in the manufacturer’s buffer binds to the protein. Fluorescent dye then binds to the sodium dodecyl sulfate micelles, and dye fluorescence as a measure of protein concentration is quantified by laser. We used the manufacturer’s gel and gel stains and primed the chip with gel according to the manufacturer’s instructions. Each albumin sample (66 kD) was mixed with the supplied sample buffer and a CA (44 kD) internal calibrator [mixture components: 20 μL sample (urine or bovine albumin), 20 μL CA (60 mg/L), 8 μL supplied sample buffer, 312 μL HPLC-grade water]. We loaded 6 μL from this total mixture in a single well of a 10-well chip, conducted electrophoresis without adding β-mercaptoethanol or heating, and compared results to the supplied sizing ladder to yield an approximate relative size under the nondenaturing conditions.

**IMMUNOASSAY**

Microalbuminuria from patient urine was quantified on a Synchron LX®20 Pro (Beckman Coulter) with generally accepted protocols.

**PURIFIED PROTEINS AND REAGENTS**

Bovine serum albumin (BSA), CA, human α₁-acid glycoprotein, human α₁-antitrypsin, human holo-transferrin, sodium chloride, urea, glucose, and β-hydroxybutyrate were acquired from Sigma-Aldrich. Erythrocytes were obtained from excess clinical specimens (VA San Diego Healthcare System). Erythrocytes were lysed by high-speed vortex mixing; and erythrocytes and hemoglobin were quantified via dipstick (Roche Diagnostics GmbH).

**URINE SPECIMENS**

Excess samples were obtained at the VA San Diego Healthcare System from patients who had submitted their urine for microalbuminuria testing by immunoassay. Patient identities were not divulged, and confidentiality was respected at all times. This protocol was approved by the University of California Human Research Protection Program for use of existing tissue (IRB030627).

**STATISTICAL ANALYSIS**

Best-fit polynomial trendlines were generated with Microsoft Office Excel 2003 and GraphPad Prism version 4.03, and the Bland-Altman graph by GraphPad Prism version 4.03.

**Results**

**SENSITIVITY AND LINEARITY OF ALBUMIN DETECTION BY CHIP ELECTROPHORESIS**

Variable concentrations of BSA were assayed by chip electrophoresis to assess sensitivity and linearity. Purified BSA was used as a surrogate for human albumin because the 2 proteins have similar molecular masses of ~66 kD (23, 24). Additionally, BSA is a readily available and inexpensive reagent that most laboratories can obtain. CA [molecular mass ~44 kD (25, 26)], was added to each BSA sample to serve as an internal calibrator. The CA concentration was kept constant (60 mg/L) for each BSA sample. Then, each BSA concentration plus CA was electrophoresed without adding a reducing agent.

A single electropherogram of BSA in relation to CA is shown in Fig. 1. CA was detected before BSA, which has a higher molecular mass. A sizing ladder confirmed the molecular mass of BSA relative to CA (data not shown). Additionally, the lowest detectable BSA concentration was 5 mg/L.

The ratio of the BSA peak area to the CA peak area was calculated for each well and plotted against the known BSA concentration (Fig. 2). Although the result was nearly linear, a polynomial gave the best fit. This trendline generated from 4 individual chips demonstrates correlation from 5 to 300 mg/L (R² = 0.9997). This best-fit polynomial was used to calculate future albumin concentrations.

**PRECISION OF MICROALBUMINURIA DETECTION BY CHIP ELECTROPHORESIS**

Assessment of precision was conducted with patient urine. Three specimens were characterized first by immunoassay: low, <20 mg/L; intermediate, 90.34 mg/L; and high, 204 mg/L. Then, each of these urine specimens was assayed 10 times on a single 10-well chip, and this procedure was conducted on 3 different chips to identify the intrachip and interchip variations, respectively (Table 1). The urine albumin/CA ratios were calculated as described earlier. Regarding the variation within a single chip (intrachip), the CVs for all 3 urine specimens were 3%–13%. For the variation among different chips (interchip), the CVs were 7%–11% for all 3 urine samples.

**COMPARISON OF BSA AND HUMAN URINARY ALBUMIN**

We compared the electrophoretic profiles of BSA and human urinary albumin. The system software uses the electropherogram data and creates a virtual electrophoretic gel (Fig. 3). Under the nonreducing conditions of

---

³ Nonstandard abbreviations: CA, chicken albumin; BSA, bovine serum albumin
the chip electrophoresis, the mobilities of BSA and albumin from patient urine were identical (molecular mass ∼66 kD) \((23, 24)\). The CA (molecular mass ∼44 kD) clearly resolves away from the BSA and human albumin. Additionally, in the patient urine sample, other nonalbumin proteins were visualized and did not migrate with CA.

**Potentially Interfering Agents**

In urine, a variety of molecular analytes \((27)\) and proteins \((21)\) could interfere with the resolving ability of the chip electrophoresis system. Therefore, we added several different analytes to BSA samples and their CA internal calibrators to identify any interference during electrophoresis.

We added various substances commonly observed in urine from patients with diabetes and renal failure to BSA (60 mg/L) and CA (60 mg/L) for chip electrophoresis and then calculated BSA/CA peak ratios, SD, and CVs. The results were as follows [analyte, tested range, mean BSA/CA ratio (SD), CV]: sodium chloride, 0–500 mmol/L, BSA/CA = 1.11 (0.09), 8%; glucose, 0–10 g/L, BSA/CA = 0.98 (0.03), 4%; urea, 0–62.5 g/L, BSA/CA = 0.86 (0.03), 4%; β-hydroxybutyrate, 0–62.5 g/L, BSA/CA = 1.11 (0.13), 12%; intact erythrocytes, 0 to 3+ (dipstick), BSA/CA = 1.05 (0.10), 9%; lysed erythrocytes, 0–3+ (dipstick), BSA/CA = 0.99 (0.04), 4%. The numbers of chips tested per analyte are as follows: sodium chloride \((n = 4\) chips), glucose \((n = 2\) chips), urea \((n = 2\) chips),

**Table 1. Intrachip and interchip variations in detecting microalbuminuria.**

<table>
<thead>
<tr>
<th>Urine Microalbuminuria</th>
<th>Mean UA/CA Ratio (SD)*</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intrachip Variation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt;20 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chip 1: 0.22 (0.01)</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Chip 2: 0.23 (0.01)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Chip 3: 0.19 (0.02)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Intermediate (90.34 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chip 1: 2.23 (0.15)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Chip 2: 2.29 (0.14)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Chip 3: 2.08 (0.12)</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>High (204 mg/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chip 1: 4.99 (0.37)</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Chip 2: 5.85 (0.77)</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Chip 3: 5.40 (0.21)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>Interchip Variation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt;20 mg/L)</td>
<td>0.21 (0.02)</td>
<td>9</td>
</tr>
<tr>
<td>Intermediate (90.34 mg/L)</td>
<td>2.20 (0.16)</td>
<td>7</td>
</tr>
<tr>
<td>High (204 mg/L)</td>
<td>5.41 (0.61)</td>
<td>11</td>
</tr>
</tbody>
</table>

Microalbuminuria was initially calculated by immunoassay to identify low, intermediate, and high specimens. Intrachip variation: For each 10-well chip, a urine specimen was assayed 10 times to assess the intrachip variation. Three chips were analyzed for each low, intermediate, and high specimen. Interchip variation: \(n = 3\) separate chips per urine specimen (low, intermediate, high specimens).

*UA, urine albumin.
β-hydroxybutyrate (n = 1 chip), intact erythrocytes (n = 1 chip), lysed erythrocytes (n = 1 chip). Despite the broad concentration ranges of the different analytes, the CV interval was only 4%–12%, which was still within the previously identified intrachip variations (Table 1.).

Sviridov et al. (21) demonstrated how urinary nonalbumin proteins (i.e., α1-acid glycoprotein, α1-antitrypsin, transferrin) coeluted with albumin during size-exclusion HPLC quantification, thereby causing an overestimation of the albumin concentration. Therefore, we investigated whether nonalbumin proteins would also coelute with BSA or CA in chip electrophoresis. We found that α1-acid glycoprotein (Fig. 4A), α1-antitrypsin (Fig. 4B), and transferrin (Fig. 4C) were resolved from BSA and CA in chip electrophoresis and did not interfere with the BSA quantification.

Comparison of Microalbuminuria Detection by Immunoassay versus Chip Electrophoresis

Osicka et al. (16, 17, 20) characterized the presence of nonimmunoreactive albumin in urine and showed that immunoassays underestimated actual total albumin concentration. Therefore, we compared the microalbuminuria concentrations for the same urine specimen detected by conventional immunoassay vs chip electrophoresis (Fig. 5). The generated best-fit polynomial between immunoassay and chip electrophoresis was nearly linear (Fig. 5A), yielding a correlation of $R^2 = 0.885$. Consistent with the findings of Osicka et al., the urine albumin concentrations enumerated by immunoassay were underestimated. Chip electrophoresis detected 3%–145% (mean 51%) more microalbuminuria than the immunoassay.

We also used the Bland-Altman method to compare chip electrophoresis to immunoassay (Fig. 5B). The resulting graph indicated that all except 2 of the data points (n = 40) fell within the 95% limit of agreement. The plot also demonstrates that the chip electrophoresis microalbuminuria values are not merely a fixed increase in the immunoassay values.

Discussion

We developed a chip electrophoresis assay as an alternative method for quantifying total microalbuminuria. It is important to note that selection of the appropriate internal calibrator is of paramount importance. The use of the chip manufacturer’s provided markers or other arbitrary proteins did not enable us to obtain accurate results. For example, use of the manufacturer’s markers to quantify the BSA concentrations shown in Fig. 2 yielded results that were only 24%–54% of the true BSA concentration (data not shown). This low recovery by the manufacturer’s semiquantitative assay prompted us to seek a more accurate and quantitative method by introducing an internal calibrator.

The similar physicochemical characteristics of CA and human albumin presumably allowed reproducible and associated responses, and the 22-kD difference in molecular weight enabled these 2 albumins to resolve easily, which made CA an ideal internal calibrator. In addition, CA does not comigrate with other urinary proteins (Fig. 3), and thereby interference with quantification is
avoided. Finally, the similar electrophoretic mobilities of BSA and human albumin (Fig. 3) permitted the use of BSA as a surrogate for human albumin during the sensitivity and precision testing.

As a result of our modifications, we determined that chip electrophoresis has a greater sensitivity (5 mg/L) than the tested immunoassay (20 mg/L detection limit), with linear detection \( (R^2 = 0.9997) \) (Fig. 2). The imprecision, as measured by the intrachip and interchip variations, indicated CVs of 3%–13% for urines with microalbuminuria concentrations up to ~200 mg/L (Table 1).

Given that urine may include a variety of small molecules, particularly in samples from persons with diabetes (i.e., ketone bodies, urea), it was imperative to see if such analytes interfered with the resolving capacity of the chip. Chip precision was not altered in any appreciable manner with the addition of these agents.

The 3 main urinary proteins that Sviridov et al. (21) reported as coeluting with albumin via size-exclusion HPLC did not coelute with the CA internal calibrator or BSA via chip electrophoresis (Fig. 4). These nonalbumin proteins were resolved from the albumins. Chip electrophoresis consistently detected more albumin in patient urine than the immunoassay (mean, 51% more) (Fig. 5A), and the values generated by chip electrophoresis were not merely a fixed percentage higher than those from immunoassay (Fig. 5B). In aggregate, our data indicate that chip electrophoresis identifies immunoreactive and nonimmunoreactive forms of albumin and that the nonalbumin proteins (α1-acid glycoprotein, α1-antitrypsin, and transferrin) do not interfere with quantification.

The current study characterized the performance of chip electrophoresis for the microalbuminuria concentration interval of 5–300 mg/L. Although further studies with BSA with concentrations up to 2000 mg/L still indicated good linear correlation \( (R^2 = 0.997, \text{data not shown}) \), the precision appeared to decrease with greater albumin concentrations. Therefore, chip electrophoresis used with our sample preparation scheme may be best suited to detect urinary albumin at concentrations <300 mg/L.

Although we attempted to identify the limits of the chip methodology, we recommend the use of a generally accepted urine dipstick screen for an initial evaluation of urine. Urine specimens <1+ by dipstick warrant assay by chip electrophoresis. Specimens that are ≥1+ for protein or blood likely have renal problems in excess of that indicated by the presence of microalbuminuria. Specimens ≥1+ for protein are no longer microalbuminuric, and at that point the amount of nonimmunoreactive urinary albumin likely would not have the same prognostic implication as that for microalbuminuria.

Other appealing features of the chip system are the ease of use and relatively fast throughput. Adequately trained personnel could assay 30 urine specimens (3 chips) in ≤2 h (~15 samples per h). The majority of this time was consumed by the automated electrophoresis (30 min per chip).

We found that avoiding the use of reducing conditions (i.e., addition of β-mercaptoethanol) and heat denaturation saved time and labor and also sharpened peak resolution (data not shown). Circumventing reducing conditions avoids fragmentation of the nonimmunoreactive albumin (17) and yields greater accuracy. The absence of such conditions, however, may allow certain proteins to bind together, thereby affecting the protein mobility during electrophoresis. For example, α1-acid glycoprotein (~40 kDa) is suspected to have trimerized in nondenaturing electrophoresis (Fig. 4). Interestingly, the α1-antitrypsin (~52 kDa) and holo-transferrin (~80 kDa) were not affected. Such effects should be taken into account when analyzing proteins under the assay conditions outlined in the Materials and Methods section.

---

**Fig. 5.** (A) microalbuminuria in patient urine as detected by immunoassay vs chip electrophoresis \((n = 40\) patient urines). Chip electrophoresis detected 3%–145% (mean 51%) more microalbuminuria than the immunoassay. (B) Bland-Altman graph comparing microalbuminuria detection by chip electrophoresis vs immunoassay \((n = 40\) patient urines). The mean microalbuminuria values by chip electrophoresis and immunoassay are depicted on the x-axis. The ratio of the microalbuminuria values by chip electrophoresis to immunoassay is depicted on the y-axis. The dotted lines represent the 95% limits of agreement (0.92–2.11). The bias was 1.51.
Because of the presence of nonimmunoreactive forms, urine samples from diabetic patients may contain more albumin than conventional immunoassays can detect (28). Comper et al. (29) observed that HPLC could detect microalbuminuria earlier than immunoassay in patients with diabetes, thus allowing time for therapeutic intervention before frank diabetic nephropathy developed. Likewise, chip electrophoresis, because of its greater sensitivity, would detect microalbuminuria earlier than immunoassay. In addition, compared with size-exclusion HPLC, chip electrophoresis has advantages of ease of use, higher throughput, and avoidance of interference by coeluting urinary proteins (i.e., α1-acid glycoprotein, α1-antitrypsin, and transferrin).

In conclusion, chip electrophoresis serves as a sensitive method for detecting immunoreactive and nonimmunoreactive forms of microalbuminuria. Chip assay is not affected by the same interferences as other methods and therefore is more accurate. In the future, establishing microalbuminuria reference intervals with this technique would have valuable clinical importance, especially for patients with diabetes and cardiovascular disease (28).

We gratefully acknowledge Bio-Rad Laboratories for their material support of the Expersion™ Automated Electrophoresis System. We also thank Dr. Robert L. Fitzgerald and Dr. Suchitura Pandey for their thoughtful insights and discussions. D.H. has previously received a consulting fee from Bio-Rad Laboratories.

References