Reliability of Urinary Albumin, Total Protein, and Creatinine Assays after Prolonged Storage: The Family Investigation of Nephropathy and Diabetes

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Background and objectives: This study investigated the effect of long-term storage at −70°C on urinary albumin, protein, and creatinine measurements in the Family Investigation of Nephropathy and Diabetes, a multicenter study designed to identify genes for diabetic nephropathy.

Design, setting, participants, & measurements: Spot urine samples were collected at eight centers and shipped overnight on ice packs to a central laboratory. Samples were aliquotted and frozen at −70°C for a median of 8 d before initial assay. As part of quality control procedures to determine interassay variability, 351 replicate samples were retrieved from storage at −70°C after a median storage time between original and quality control analyses of 126 d (range 28 to 869 d). Freezer time was characterized as the difference in days between the initial assay and quality control assay. Percentage difference [(quality control/original/original) × 100%] between samples was regressed on storage time and adjusted for original value, age, race, gender, hypertension, and diabetes.

Results: After adjustment, freezer time per 30 d was associated with small decreases in percentage difference of urinary albumin (0.25%, P = 0.02), total protein (0.23%, P = 0.02), and albumin-to-creatinine ratio (0.34%, P = 0.001). Urinary creatinine levels were not affected by freezer time (P = 0.25).

Conclusions: Measurements of urinary albumin, total protein, and albumin-to-creatinine ratio are minimally affected by storage at −70°C for approximately 2.5 yr. Prolonged storage results in small decreases of urinary albumin and protein that do not substantially affect phenotype classification of overt renal disease.


Urinary albumin excretion is a sensitive and early marker of diabetic nephropathy, other forms of renal dysfunction, and endothelial permeability throughout the vascular tree. Minimally elevated levels of urinary albumin excretion, commonly termed “microalbuminuria,” predict progression of kidney disease, development of cardiovascular disease, and total mortality (1,2). For these reasons, urinary albumin excretion is being estimated increasingly frequently in observational studies of renal and cardiovascular disease, often on frozen urine specimens that have been stored for prolonged periods. Storage of urine at −70°C is recommended when assay of freshly voided specimens is not feasible (3). Stability of quantitative measures of albumin in urine stored at −70°C for >180 d, however, is unknown. In addition, it is not known whether urinary proteins are stable across a wide range of chronic kidney disease conditions resulting in microalbuminuria to nephrotic-range proteinuria. We addressed the reproducibility of determinations of urinary albumin excretion using data from a study that was designed to identify genes that are associated with diabetic nephropathy and other forms of renal disease: The Family Investigation of Nephropathy and Diabetes (FIND) (4,5). As part of the quality control (QC) procedures for this study, urine specimens were reassayed for albumin level after being stored for up to 900 d.
Concise Methods

Overall Study Design

FIND is a multicenter study that enrolls participants into two study protocols at eight centers. The two protocols are a family-based linkage approach and a case-control study using a mapping by admixture linkage disequilibrium approach (MALD). The FIND family protocol is designed to perform linkage analysis in families of probands who have diabetic nephropathy and have either two living parents or a sibling with diabetes. In contrast to the family protocol, the MALD protocol uses a case-control design and enrolls individuals with African-American or Mexican-American ancestry. In the African-American MALD protocol, probands may have nephropathy as a result of causes other than diabetes; the spouse or partner and, when available, their child, are also recruited. In the Mexican-American MALD study, only population control subjects with diabetes are used. The design of the FIND study resulted in participants with a wide range of kidney function being screened for enrollment. More detailed overviews of the FIND study appear elsewhere (4,5). Demographic variables and history of diabetes or hypertension were determined by self-report from participant questionnaire at enrollment into FIND.

Laboratory Assays

The eligibility criteria for probands, normal control subjects, and affected and unaffected siblings in FIND are based, in part, on urine albumin excretion. Because the amount of albumin in the urine varies with urine concentration, the ratio of albumin to creatinine is used as the estimate of albumin excretion. Probands and family members are classified by urine albumin (mg) to creatinine (mg) ratio (UAC ratio) as normal (UAC ratio <0.03), microalbuminuric (UAC ratio 0.03 to <0.3), or overtly albuminuric (UAC ratio ≥0.3). For assessment of phenotype, random urine specimens are obtained from potential participants and sent on an ice pack via overnight mail to a central processing facility at the Laboratory of Genomic Diversity of the National Cancer Institute at the Frederick Medical Research Center (Frederick, MD). Urine specimens are spun, aliquotted, and stored at −70°C before being batch-shipped to a central phenotyping laboratory in Washington, DC. Aliquots are also sent to the Genetic Analysis and Data Coordinating Center at Case Western Reserve University (Cleveland, OH), where they are stored at −70°C. For assessment of the reproducibility of the urinary laboratory assays, approximately 5% of samples are re-sent to the central phenotyping laboratory from those archived at the Genetic Analysis and Data Coordinating Center. These duplicate QC specimens are selected on the basis of the amount of urine remaining for individual participants and are sent in a blinded manner. This report includes data from 353 replicate samples. The original specimens were collected from January 17, 2001, to July 28, 2003, and assayed from May 10, 2001, to August 6, 2003. The QC specimens were assayed from August 6, 2002, to November 18, 2004. Urine specimens used in the study went through one freeze–thaw cycle before the original measurement.

At the central phenotyping laboratory, urine specimens are thawed and mixed, and albumin is measured by the Roche/Hitachi 717 (F. Hoffmann-La Roche Ltd., Diagnostics Division, Basel, Switzerland) analyzer using the immunoturbidimetric method, creatinine by the VITROS 250 CREA slides (VITROS; Ortho-Clinical Diagnostics, Raritan, NJ) using the two-point system, and total protein by the VITROS 250 UPRO slides (VITROS; Ortho-Clinical Diagnostics) using the colorimetric method. The analytic coefficient of variation of the urinary assay measurements during the study period ranged from 1.6 to 6.6% for albumin, 3.3 to 6.1% for protein, and 0.0 to 8.4% for creatinine.

A total of 21 participants had urine samples split for shipment to the central laboratory as well as local analyses of the fresh urine for albumin and creatinine. This subset was analyzed to compare the analytes measured between fresh and the first-frozen specimens. The urine albumin was measured by a double antibody albumin in vitro diagnostic (DPC, Los Angeles, CA) using a Wallac Wizard 1470 Automatic Gamma Counter machine, and urine creatinine was measured by picric acid colorimetric assay (Raichem, San Diego, CA) using a Cobas-Mira machine (Roche).

The FIND protocol was reviewed and approved by the institutional review board of Case Western Reserve University and all other participating centers. All patients were informed and consented to the study before participation.

Statistical Analyses

Initially, distributions of values were examined for outliers and for normality. Mean, SD, and quartile ranges were calculated for QC and original samples as well as for the percentage difference between the samples. Percentage difference of each analyte was defined as the difference between QC and original values divided by the original value times 100% or [(QC − original)/original] × 100%. Freezer storage time (in months) was calculated as the difference between the QC assay date and the original assay date.

For assessment of whether measurements of QC samples were different from those of original samples, a one-sample t test was performed to test the null hypothesis that percentage difference for each analyte was equal to zero. The impact of differences between original and QC values on affection status change (normal, microalbuminuric, and overtly albuminuric) was assessed by cross-tabulating the affection status as defined by original values against those defined by QC values.

For assessment of the association between percentage difference between QC and original samples and the time elapsed between the two measurements, linear regression models were constructed with percentage difference for each analyte as the dependent variable and the freezer storage time, in months, as the main predictor variable. The t test was used to assess the significance of the association between percentage difference for each analyte and freezer storage time. For assessment of whether the association between percentage difference for each analyte and freezer storage time differed by clinical and demographic factors such as age, diabetes status, and hypertension status, stratified analyses were performed. Interaction terms were included in multiple linear regressions to assess the significance of any effect modification. When no significant effect modification was observed, pooled analyses were performed while adjusting for these clinical and demographic variables. Regression diagnostic plots were performed and demonstrated a good fit of the data. Bland-Altman plots of the difference between the QC and original assays by the average value of the two assays were examined to determine whether any difference between the assays varied by level of albumin or protein excretion (6,7). In addition, correlation and regression analyses were conducted to compare urine albumin, creatinine, and the ratio in both fresh and the first-frozen samples among a subset of the study population. Statistical analyses were conducted using Stata 8 (Stat Corp., College Station, TX). Two-tailed α levels of ≤0.05 were used for all analyses.

Results

Table 1 shows the characteristics of the 351 participants who contributed data to this analysis. The average age of the study participants was 55 yr, 40% were men, 59% had a history of hypertension, and 65% had a history of diabetes. The median storage time between date of initial collection and date of original analysis was 8 d (range 3 to 35 d). The median storage time between original and QC analyses was 126 d (range 28 to 869 d).

Descriptive statistics of the original and QC assays for urinary
albumin, creatinine, total protein, and UAC ratio are given in Table 2. Original urinary albumin measurements ranged from 5.7 to 5460.0 mg/L, reflecting the wide eligibility criteria for the study and the lower limit of sensitivity for the assay. Urinary total protein also varied widely, from 4.9 to 1340.0 mg/dl. The range of the original UAC ratio was from a low of 0.0025 to a high of 7.95. Urinary creatinine ranged from 11 to 412 mg/dl. Percentage differences between original and QC assays were small for all analytes, <2.5%. These small differences were not statistically significantly different from zero except for UAC ratio ($P = 0.02$ for albumin; $P = 0.92$ for creatinine; $P = 0.32$ for total protein; $P = 0.02$ for UAC).

Figure 1 shows the percentage difference between original and QC analyses versus the number of months in storage at $-70^\circ$C. The regression coefficients of percentage difference on freezer time are given in Table 3. For each 30 d in the freezer, there was a decrease of 0.27% in urinary albumin level ($P = 0.009$), 0.17% in urinary total protein ($P = 0.07$), and 0.33% in UAC ratio ($P < 0.001$). After multivariate adjustment, the percentage decrease remained significant for urinary albumin, total protein, and the UAC ratio. In both unadjusted and adjusted analyses, freezer time did not affect replicability of urinary creatinine measurements. In addition, gender, race, age, diabetes, and hypertension were not associated with percentage difference in replicate samples for any of the urinary analytes (data not shown). Also, the percentage differences of urinary albumin ($P = 0.6$), creatinine ($P = 0.5$), total protein ($P = 0.1$), and UAC ratio did not vary between patients with diabetes compared with patients without diabetes.

Figure 2 presents the difference in urinary analytes between the QC and original urine sample compared with the wide range of mean urine excretion of albumin and protein seen in our study population. These plots demonstrate that only at high levels of urine albumin or total protein excretion are there a few values that exceed 2 SD from zero.

Table 4 presents change in affection status, defined by UAC ratio, on repeat measurement after storage. Overall, there was good agreement between affection categories with a $\kappa$ of 0.93. A total of 209 patients were initially classified as having normal urinary albumin concentration, and only five (2%) were reclassified as having microalbuminuria after the QC assay. In the 75 patients with evidence of microalbuminuria at original assay, nine (12%) changed status after the QC assay, five of whom (6%) were considered to have normal UAC ratio. The remaining four (5%) were defined as having overt albuminuria. All patients who were reclassified had values close to the cut point for difference categories. None of the 59 patients with overt albuminuria at baseline was reclassified.
Among the 21 participants with measurements conducted on both fresh and frozen urine, the correlation coefficient was 0.8 for urinary creatinine, 0.9 for urinary albumin, and 0.99 for the UAC ratio. By regression analyses, for every 1-mg/ml increase of urine albumin in the fresh specimen, there was a corresponding 0.86-U increase in urine albumin from the frozen specimen (β coefficient 0.86; 95% confidence interval 0.61 to 1.1) and similar findings in urine creatinine. Figure 3 shows a scatter plot with the raw values comparing fresh urine UAC ratio with the frozen urine UAC ratio.

**Discussion**

This study demonstrates that estimates of urinary albumin, total protein, and UAC ratio decrease modestly after 2.5 yr in storage at −70°C. Urinary creatinine, however, is stable after

**Table 3. Association of freezer time (β) and percentage difference in replicates by linear regression analyses**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Percent Difference per 30 D (β)</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unadjusted</td>
<td>−0.27</td>
<td>0.009</td>
<td>−0.48 to −0.07</td>
</tr>
<tr>
<td>multivariate adjustment</td>
<td>−0.25</td>
<td>0.023</td>
<td>−0.46 to −0.03</td>
</tr>
<tr>
<td>Creatinine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unadjusted</td>
<td>0.08</td>
<td>0.21</td>
<td>−0.05 to 0.21</td>
</tr>
<tr>
<td>multivariate adjustment</td>
<td>0.08</td>
<td>0.25</td>
<td>−0.06 to 0.21</td>
</tr>
<tr>
<td>Total protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unadjusted</td>
<td>−0.17</td>
<td>0.07</td>
<td>−0.36 to 0.01</td>
</tr>
<tr>
<td>multivariate adjustment</td>
<td>−0.23</td>
<td>0.02</td>
<td>−0.43 to −0.04</td>
</tr>
<tr>
<td>UAC Ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>unadjusted</td>
<td>−0.34</td>
<td>&lt;0.001</td>
<td>−0.53 to −0.16</td>
</tr>
<tr>
<td>multivariate adjustment</td>
<td>−0.34</td>
<td>0.001</td>
<td>−0.53 to −0.14</td>
</tr>
</tbody>
</table>

*Multivariate analyses adjusted for original value, age, race, gender, hypertension, and diabetes status.
prolonged storage at −70°C. These findings extend the results of previous studies in several ways (3,8–10). Most important, the length of time between the original and repeat assays is much longer than in previous investigations of this issue, which studied only frozen specimens up to 6 mo at −70°C or up to 24 mo at −20°C (11). In addition, the effect of freezer time was tested across a wide range of urinary albumin excretion, demographic characteristics, and severity of kidney disease.

Several previous studies examined the stability of urine albumin determinations in stored specimens. In a study of children and adolescents with diabetes from the United Kingdom, freezing at −20°C led to marked underestimation of the urinary albumin excretion compared with assays on fresh specimens (10). Similarly, a study from Japan found that storage of specimens from 29 patients with diabetes at −20°C for only 9 wk resulted in a 50% decrease in albumin estimates but remained unchanged in −80°C (9). In a study of 95 children with type 1 diabetes, storage for 6 to 8 mo at −20°C led to lower estimates of urinary albumin excretion compared with storage at −70°C (3). In our study, there was a decrease in urinary albumin

\[ \text{Figure 2. Bland-Altman plots of difference in urinary analytes versus mean urinary concentration of urinary analytes at } -70\,\text{°C. (A) Alumin. (B) Creatinine. (C) Total protein. (D) UAC ratio. The dashed lines denote 2 SD above and below the mean difference of zero.} \]

\[ \text{Table 4. Change in affection status of UAC ratio after QC analyses and prolonged freezer time}^a \]

<table>
<thead>
<tr>
<th>Original UAC Ratio</th>
<th>QC UAC Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>&lt;0.03 (n = 209)</td>
<td>204</td>
</tr>
<tr>
<td>0.03 to 0.3 (n = 75)</td>
<td>5</td>
</tr>
<tr>
<td>&gt;0.3 (n = 59)</td>
<td>0</td>
</tr>
</tbody>
</table>

\[ ^a \text{Percentage agreement} = 96\%; \kappa = 0.93. \]

\[ \text{Figure 3. Scatter plot of UAC ratio from the fresh urine compared with the UAC ratio from the frozen urine among a subset of Family Investigation of Nephropathy and Diabetes (FIND) participants.} \]
concentration over time despite having storage at \(-70^\circ\text{C}\). For example, the percentage decrease of urinary albumin was 0.25\% over 30 d and 6\% over 2 yr. This percentage decrease is well within laboratory variability of the urine albumin assay measurement (12). Similarly, the UAC ratio was found to decrease by 0.32\% over 30 d and 7.7\% over 2 yr. The lower estimation of albumin excretion and UAC ratio after years of storage, however, did result in reclassification of phenotype for patients with normoalbuminuria or microalbuminuria. It did affect the classification of 12\% of patients who were defined as having microalbuminuria at first measure, likely as a result of UAC ratios that were close to the cut points of UAC categories. Our study is the first to show that urinary total protein measurements decrease with frozen storage. Similar studies of the effect of prolonged storage and freezer time on urinary total protein are not available for comparison. Overall, measurements of urinary albumin and total protein are minimally affected by storage at \(-70^\circ\text{C}\) for approximately 2.5 yr, and these small changes may affect reclassification only of patients with normal or low levels of albuminuria or proteinuria. These findings are especially important in light of a recent publication of data from the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study, in which the changes in urine albumin degradation altered the relationship with mortality. In this study, the urine was stored at \(-20^\circ\text{C}\), and it should be recommended that all urine be stored at \(-70^\circ\text{C}\) (13).

Urinary creatinine levels are reported to be stable in short-term storage for up to 28 d at room temperature and \(-4^\circ\text{C}\) and \(-20^\circ\text{C}\) (14,15). Prolonged storage for up to 6 mo at \(-20^\circ\text{C}\) has also shown minimal effect in urinary creatinine levels (3.16). In our study, after prolonged storage at \(-70^\circ\text{C}\), there was no significant change in urinary creatinine concentration up to 900 d. The stability of urine creatinine is important because urinary creatinine concentration is often used as a reference for urinary parameters.

Methods of measuring urinary analytes are not standard across laboratories in the United States or Europe (17,18), and our results may not be generalizable to all methods available; however, the immunoturbidimetric assay that was used to measure urinary albumin in this study agrees well with immunonephelometric assays and RIA (17–19). No studies on the interassay variability of urinary creatinine and total protein were found. The abundance of studies on urinary albumin measurement reflects the importance of urinary albumin as a screening tool in clinical and research settings.

FIND has several strengths in addressing the stability of urinary analytes over time. The sample size of our study was much larger than that in previous studies, allowing much greater statistical power to detect associations and to examine agreement between original and replicate measures in subgroups. Because of its study design, the range of albumin and protein excretion was very broad, extending from the lower limit of the assays to the overtly albuminuric and proteinuric range. In addition, the demographic makeup and clinical characteristics of the study population were diverse. These study characteristics enhance the generalizability of our results to other populations and allowed testing of characteristics that were associated with disagreement between the original and QC assays. Results of the study were consistent across demographic and clinically important subgroups, adding to confidence in the study results. Because measurements were not done routinely on fresh, unfrozen specimens, the effect of a freeze-thaw cycle on the assays cannot be determined, an important limitation of our study, because frequent freeze-thaw cycles could result in further degradation of proteins (20). We were, however, able to demonstrate in a small subset of the study population that there were no systematic biases comparing the analyses of fresh urine with the urine that had been frozen, which differed from the PREVEND study (13). In addition, we cannot account for specimen-handling differences by center or by laboratory personnel. There was, however, standardization of collection tubes and storage of specimen collection, which have been reported to alter degradation of proteins (20). Also, samples were not randomly chosen by the urine albumin or protein values; however, there seemed to be similar values of QC and the original sample among the higher and lower range of urine albumin excretion. More recently, analyses of the PREVEND study samples have shown that higher urine pH may also prevent protein degradation in frozen specimens that are stored at \(-20^\circ\text{C}\) (21). We did not measure urine pH in FIND, and this finding will need to be considered in planning future studies.

Conclusions

This study shows that prolonged storage of urine specimens at \(-70^\circ\text{C}\) results in small decreases in urinary albumin and total protein that do not substantially affect phenotype classification of overt renal disease.

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Disclosures
None.

References