Decreases in Albumin/Creatinine and N-Acetylglucosaminidase/Creatinine Ratios in Urine Samples Stored at -20 °C

Susan E. Manley, Mary E. Burton, Karen E. Fisher, Carole A. Cull, and Robert C. Turner

The effects of storage for 6 months or 2 years at -20 °C were studied in urine samples from Type II diabetic patients by assaying albumin by immunoturbidity, N-acetylglucosaminidase (EC 3.2.1.30) by methoxynitrovinylphenol release, and creatinine by the Jaffé method. There were significant decreases (P < 0.001) in albumin/creatinine ratios from 1.14 (0.63–2.98) to 0.83 (0.32–2.12) g/mol (median + interquartile ranges) after 6 months (n = 97), and from 1.64 (0.74–5.72) to 1.60 (0.37–4.54) g/mol after 2 years (n = 89). The percentage of samples with results below the detection limit of the albumin assay (2 mg/L) increased from 5% to 21% after 6 months and from 0% to 34% after 2 years. N-Acetylglucosaminidase/creatinine ratios decreased (P < 0.001) from 520 (356–832) to 380 (263–695) U/mol after 6 months and from 520 (330–865) to 258 (82–462) U/mol after 2 years. The effect of storage was greater in samples with concentrations in the normal range (<2.5 g/mol for albumin/creatinine, <500 U/mol for N-acetylglucosaminidase/creatinine). Samples with albumin concentrations more than twice the normal range were still detected as abnormal after storage at -20 °C; e.g., 18% were >5 g/mol (albumin/creatinine) initially, with 17% >5 g/mol after 6 months of storage. We therefore recommend storage of urine samples at 4 °C for no longer than 7 days before assay.

Additional Keyphrases: diabetes · Immunoturbidimetry · enzyme activity · sample handling · variation, source of · renal function

Monitoring the excretion of albumin (1–4) and N-acetylglucosaminidase (EC 3.2.1.30) (5, 6) in urine provides information on the progression of renal dysfunction in diabetic patients. Increased albumin excretion is thought to indicate glomerular damage in the kidney, whereas N-acetylglucosaminidase indicates tubular damage, this enzyme being found in the lysosomes of kidney tubular cells. Both albumin and N-acetylglucosaminidase can be measured in single urine samples with creatinine also measured to allow for estimation of dilution of urine (7, 8). All three measurements are precise, unlike the timing for 24-h collections, which can be unreliable.

For many research studies urine samples are stored at -20 °C before assay. Various reports of studies involving 50–100 samples show significant decreases in urine albumin after storage at -20 °C for 7 days (9); 2, 8, and 24 weeks (10); or 2 and 6 months (11), but no change in albumin concentration after storage at 4 °C for 8 weeks (10). Decreases after storage for 6 weeks at -20 °C in samples at low concentrations have been reported as measured with a sensitive enzyme-linked immunosorbent assay (ELISA) (12).

Some studies with small numbers of samples (<20) showed no effect of storage at -20 °C for 10 days (13) or 1 month (14), but did show losses in some samples after 2 months (15). Townsend et al. (16, 17) suggested that adjusting the pH of urine samples (to <5.5) might prevent the effect of storage at -20 °C on albumin.

N-Acetylglucosaminidase has been measured in urine 2 h after voiding (18), after storage for 48 h at 4 °C (19), after ≤1 month at 4 °C (20), or after an indefinite period at -20 °C (6). Enzyme activity is reportedly stable at 4 °C for 48 h (19), decreasing in activity by <5% in samples without preservative after storage for 1 month at 4 °C (20).

The purpose of this study was to investigate the effect of storage for 6 months or 2 years at -20 °C on urine concentrations of albumin, N-acetylglucosaminidase, and creatinine, and on the albumin/creatinine and N-acetylglucosaminidase/creatinine ratios.

Materials and Methods

Single urine samples were obtained from fasting Type II diabetic patients entered into the UK Prospective Diabetes Study (21). Separate sets of randomly chosen samples from patients at any visit in the study were used for both periods for the study of the effect of storage at -20 °C. Samples from newly diagnosed diabetic patients were analyzed to provide an example of a range of values found in clinical practice.
Thiomersal was added to the urine as a preservative (50 μL of 1 g/L thiomersal reagent per 6-mL tube of urine); samples were stored for as long as 7 days at 4 °C before assay. This concentration of thiomersal does not affect N-acetylglucosaminidase activity, and treated samples can be stored for up to 1 week at 4 °C (22). Samples were subsequently stored at −20 °C and reassayed after 6 months or 2 years.

Albumin was determined by an immunoturbidimetric method (23) with use of goat antihuman albumin antisem (Incstar Ltd., Winneke, Berks., UK) and a human serum protein calibrator (Dakopatts, High Wycombe, Bucks, UK). Samples were centrifuged at 1500 × g for 10 min to clear them of particulate matter and then screened with Albstix (Ames Division, Miles Labs. Ltd., Stoke Poges, Berks, UK) to indicate which samples required dilution. Quality control was monitored by using controls at three concentrations and an in-house computer package, CSTAT, applying the rules of Westgard et al. (24) for assay rejection. The lower limit of the albumin assay was 2 mg/L, and interassay CVs were 5.6% at 4.4 mg/L, 3.1% at 31.3 mg/L, and 6.5% at 136.7 mg/L. For calculations, values assayed as <2 mg/L were set to 2 mg/L. External quality control was monitored over the period of the study by the UK External Quality Assurance Scheme (UKEQAS; Queen Elizabeth Hospital, Birmingham, UK). Agreement between our results for albumin and the values designated for the specimens in UKEQAS over 2 years was high (r = 0.997), with no change in bias of the assay.

Urine N-acetylglucosaminidase was measured by the release of methoxyxynitrovinylphenol (25) (Cortec Diagnostics Ltd., Deeside, Clywed, UK), and creatinine by the endpoint Jaffé (26, 27) method. All three assays were performed on the same sample by a centrifugal analyzer (Cobas FARA; Roche Diagnostica, Welwyn Garden City, Herts., UK). Interassay CVs for N-acetylglucosaminidase were 4.7% at 3.92 U/L, 4.5% at 12.1 U/L, and 5.5% at 31.9 U/L; for creatinine, they were 6.1% at 3.3 mmol/L, 3.3% at 8.9 mmol/L, and 3.9% at 17.0 mmol/L.

Statistical methods. The statistical computer programs MINITAB STATISTICAL SOFTWARE™ and CSTAT (Oxtech, Oxford, UK), were used for calculations, and SLIDEBRITE PLUS (Advance Graphics Software Inc., Sunnyvale, CA) for plotting scattergrams and Altman–Bland difference plots (28). For comparisons between samples measured fresh and then after freezing, we used the Wilcoxon sign-rank test and chi-square tests to compare proportions. Ranges are expressed as 25–75 percentiles (interquartiles, i.e.).

Results

There were no significant differences between the initial values for albumin, N-acetylglucosaminidase, or creatinine in the two sets of fresh samples that were to be stored.

Effects of Storage

On albumin. Median albumin concentrations in urine samples frozen for 6 months or 2 years were lower (P < 0.001) than in the same samples when measured after storage for <1 week at 4 °C, the median differences being 2.6 and 6.4 mg/L, respectively (Table 1). The loss in albumin after 6 months storage was variable. Values ≤25 mg/L for fresh samples could be reduced to below the detection limit (<2 mg/L) after freezing, but this loss was not consistent and some samples showed no loss of albumin (Figure 1, top). Albumin loss as great as 50 mg/L could occur after 6 months storage of samples having higher concentrations, but proportionately the difference was smaller than for the lower-concentration samples (Figure 1, bottom). After 2 years, most samples had lost as much as 100 mg/L.

The proportion of urine samples giving results below the detection limit of the albumin assay increased from 5% in fresh samples to 21% in samples stored for 6 months (P < 0.01). In the group of samples stored for 2

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**Table 1. Effect of Storage at −20 °C on Albumin, N-Acetylglucosaminidase, Creatinine, and Albumin/Creatinine and N-Acetylglucosaminidase/Creatinine Ratios in Urine Samples from Type II Diabetic Subjects**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Fresh</th>
<th>Frozen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (mg/L)</td>
<td>12 (5–37)</td>
<td>8 (2–27)</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>10.1 (6.1–13.9)</td>
<td>10.2 (6.2–14.0)</td>
</tr>
<tr>
<td>NAGase (U/L)</td>
<td>4.92 (2.70–8.02)</td>
<td>3.85 (2.15–7.12)</td>
</tr>
<tr>
<td>Albumin/creatinine ratio (g/mol)</td>
<td>1.14 (0.63–2.98)</td>
<td>0.83 (0.32–2.12)</td>
</tr>
<tr>
<td>NAGase/creatinine ratio (U/mol)</td>
<td>520 (358–832)</td>
<td>380 (263–695)</td>
</tr>
</tbody>
</table>

NAGase, N-acetylglucosaminidase.

* Concentration measured within 1 week of collection, stored at 4 °C until assay.

b,c Significantly different from fresh values: b P < 0.001, c P < 0.05.
years, the proportion <2 mg/L increased from 0% when assayed fresh to 34% when assayed after storage at −20 °C (P < 0.001).

**On N-acetylglucosaminidase.** Urine N-acetylglucosaminidase also showed a significant decrease after 6 months' storage at −20 °C (P < 0.001), with a median difference of 0.875 U/L in samples containing up to 21.7 U/L (Table 1 and Figure 2, top). The enzyme in 80% of the samples had decreased after 6 months storage; by 2 years, 100% of samples in this range had decreased activity, with a median difference of 2.33 U/L.

**On creatinine.** Creatinine values were only slightly reduced after 6 months storage at −20 °C (P <0.05)—median difference 0.27 mmol/L—but were significantly decreased after storage for 2 years at −20 °C (P < 0.001)—median difference 1.36 mmol/L (Table 1 and Figure 3, top). There was no indication of concentration-dependent changes in the decrease after 6 months or 2 years (Figure 3, bottom).

**On albumin/creatinine ratios.** The urine albumin values can be adjusted for dilution of the urine by reference to the creatinine value. After 6 months' storage, there was a significant decrease in the albumin/creatinine ratios from median values of 1.14 g/mol (i.e. range 0.63–2.96) when fresh to 0.83 g/mol (i.e. range 0.32–2.12) after storage (Table 1 and Figure 4). This effect was most marked at the lower end of the range, where samples with a ratio when fresh of ≤2.5 g/mol, the upper limit of the normal range, could fall to zero after storage (Figure 4, bottom). Values >5 g/mol when fresh were always >2.5 g/mol after storage when frozen, but some values that were only slightly above normal (2.5–4.9 g/mol) could fall to <2.5 g/mol.

Albumin/creatinine ratios were also significantly decreased across the whole range of values by storage for 2 years (Table 1), with proportionally more effect at the lower end of the range (see Figure 4, bottom right).
However, the effect of freezing was mitigated to a certain extent by the decrease in creatinine during storage, which was constant across the range of concentrations. Samples >5 g/mol when fresh were never <2.5 g/mol after freezing.

Table 2 shows that the proportion of the patient population who had microalbuminuria (albumin/creatinine >2.5 g/mol) in fresh samples decreased when samples were measured after freezing; however, if the criterion was changed to >5 g/mol, the difference was less marked. The sensitivity of detecting values >5 g/mol was 94% for the samples stored for 6 months and 83% for those stored for 2 years (Table 2).

Samples in which the albumin levels fell below the limit of the assay on freezing (Figure 4, bottom) in all but two cases involved patients without microalbuminuria >2.5 g/mol in the fresh samples. Setting the frozen value for albumin in these samples to the lower limit of sensitivity of the assay, 2 mg/L, never induced spurious microalbuminuria >2.5 g/mol.

On N-acetylglucosaminidase/creatinine ratios. After 6 months' storage, the majority of N-acetylglucosaminidase/creatinine values were decreased, but except for two samples, values >1000 U/mol on fresh samples did not decrease to <500 U/mol, the upper range of normal, in frozen samples (Figure 5). After 2 years' storage, the decrease in N-acetylglucosaminidase/creatinine was more marked, such that four samples with fresh values >1000 U/mol had decreased into the normal range (<500 U/mol). The proportion of the subjects who had high values (being above a ratio of either 500 or 1000 U/mol) was less when measured in frozen samples (Table 2).

Clinical Ranges in Newly Presenting Type II Diabetic Patients

At diagnosis, median values for urine albumin/creatinine ratios were within the normal range (<2.5 g/mol), but urine N-acetylglucosaminidase/creatinine ratios were outside the normal range (>500 U/mol) (Table 3). The 90th percentile for albumin/creatinine ratios and the 75th percentile for the N-acetylglucosaminidase/creatinine ratios were above twice the upper limit of the normal range.

Discussion

Albumin/creatinine ratios in newly presenting Type II diabetic patients, as measured in urine samples stored at 4 °C, ranged from 0.66 to 2.83 g/mol (i.e., range), with a median of 1.2 g/mol. More than 25% of these patients could be therefore classified as having microalbuminuria as defined by an albumin/creatinine ratio >2.5 g/mol. N-Acetylglucosaminidase/creatinine ratios ranged from 412 to 1062 U/mol, with the median value of 697 U/mol being above the upper limit of normal, 500 U/mol. Over 25% of these patients had N-acetylglucosaminidase/creatinine ratios greater than twice the normal range.

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**Table 2. Effect of Urine Storage at -20 °C on Percentage of Diabetic Subjects Diagnosed with Microalbuminuria**

<table>
<thead>
<tr>
<th>Length of storage</th>
<th>Albumin/creatinine ratio</th>
<th>NAGase/creatinine ratio</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>&gt;2.5 g/mol</td>
<td>&gt;5.0 g/mol</td>
</tr>
<tr>
<td>6 months</td>
<td>Fresh* 31</td>
<td>Frozen 21</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>31</td>
</tr>
</tbody>
</table>

*As in Table 1.
*P* value of significance of difference from fresh values given in parentheses.
In many research studies, urine samples are stored frozen for months or years, and the question is whether their concentrations could be markedly affected by sample storage at -20 °C. This study shows that storing urine at -20 °C for 6 months resulted in a measurable decrease in albumin concentrations, although not all samples were affected. These decreases tended to be of the same magnitude across the range of values, so that proportionately the greatest decrease was at low values. A value for albumin below the detection limit of the assay, 2 mg/L, could be obtained for a sample that had been frozen for 6 months when values were between 0 and 30 mg/L in the fresh sample. Assay values in the normal range is markedly affected by freezing, but samples above the normal range are proportionately less affected. Samples with microalbuminuria >5 g/mol (albumin/creatinine) always remained >2.5 g/mol, the upper limit of the normal range. Thus, markedly abnormal values continued to be detected as being above normal after freezing urine samples.

Urine N-acetylglucosaminidase showed a constant decrease across the range of values after storage for 6 months at -20 °C. After 2 years, the differences were more marked, with variable loss in most samples; with a criterion of either more than the normal range, >500 U/mol, or twice this value, the sensitivity of detection was only approximately ±50% on frozen samples.

Freezing urine specimens may cause conformational changes in urinary proteins, resulting in partial precipitation, with lack of antibody recognition for albumin and loss of enzyme activity for N-acetylglucosaminidase. Urine can be stored at 4 °C for 8 weeks before assay of albumin (10), but whether this storage would affect N-acetylglucosaminidase has not been reported. In a recent review, Rowe et al. (29) suggested that no problems would be encountered if samples had been stored at -20 °C were mixed immediately before assay. However, all frozen samples were mixed and centrifuged before analyses in our study, and this did not prevent a decrease in analyte concentration when stored frozen.

Urine creatinine was relatively stable after storage for 6 months at -20 °C, but after 2 years significant decreases occurred (Figure 3, top). This decrease in measured creatinine, which was constant across the range of concentrations, could be due to variation in an analyte that interferes with the assay. Creatinine itself is thought to be stable but the endpoint Jaffe method is prone to several interferences. Storage of urine samples at -20 °C for >6 months is not recommended.

It is unlikely that the effects of storage are caused by evaporation of water from the tubes, because this would lead to increased concentrations of the analytes.

We do not know whether these decreases in albumin, creatinine, and N-acetylglucosaminidase are related to each other. Adsorption to the walls of the tubes on storage has been suggested as a possible cause (30) of decrease in albumin, which could account for the changes seen in urine proteins in this study, although any adsorption would have to be time related.

In conclusion, both albumin/creatinine and N-acetylglucosaminidase/creatinine ratios are significantly decreased after 6 months or 2 years of storage at -20 °C. These ratios are markedly affected in the normal range, and at the clinical thresholds for microalbuminuria and increased N-acetylglucosaminidase excretion. When possible, urine samples should be analyzed fresh or after storage at 4 °C. When measurement after storage at -20 °C is necessary, detection of microalbuminuria is more accurate if the criterion is set to >5 g/mol (twice the normal range for urine albumin excretion): results for <25% of newly presenting Type II diabetic patients fell into this category. The greater effect of freezing on

### Table 3. Clinical Ranges in Newly Presenting Type II Diabetic Patients as Measured in Fresh* Urine Samples

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>10%</th>
<th>25%</th>
<th>Median</th>
<th>75%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin, mg/L</td>
<td>799</td>
<td>3.0</td>
<td>6.0</td>
<td>14.0</td>
<td>29.0</td>
<td>87.0</td>
</tr>
<tr>
<td>NAGase, U/L</td>
<td>659</td>
<td>2.47</td>
<td>4.33</td>
<td>6.92</td>
<td>11.18</td>
<td>17.27</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td>810</td>
<td>4.7</td>
<td>7.2</td>
<td>10.7</td>
<td>15.4</td>
<td>19.5</td>
</tr>
<tr>
<td>Albumin/creatinine ratio, g/mol</td>
<td>792</td>
<td>0.38</td>
<td>0.66</td>
<td>1.20</td>
<td>2.83</td>
<td>7.81</td>
</tr>
<tr>
<td>NAGase/creatinine ratio, U/mol</td>
<td>655</td>
<td>315</td>
<td>457</td>
<td>697</td>
<td>1062</td>
<td>1592</td>
</tr>
</tbody>
</table>

* Defined as in Table 1.
N-acetylglucosaminidase in urine after 2 years makes assay of these frozen samples of little value, although an allowance for the smaller decrease across the range for samples stored for 6 months might be feasible.

This study was done as part of biochemical evaluation for the UK Prospective Diabetes Study, funded by the National Institute of Diabetes, Digestive, and Kidney Diseases of the U.S. National Institutes of Health; British Diabetic Association; Medical Research Council; and pharmaceutical companies, including Bristol Myers Squibb, Lilly, Novo Nordisk, Hoehst, Lipha, and Farmitalia Carlo Erba. We are grateful for the technical assistance of Robin Carter and Ted Bown, and to David Bullock and Robin Chambers for allowing us to use data from UK EQAS.

References