Apparent Loss of Urinary Albumin during Long-term Frozen Storage: HPLC vs Immunonephelometry

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Background: Urinary albumin detection by immunonephelometry is decreased by ~30% in samples that have been frozen at −20 °C. An HPLC method for assessment of urinary albumin that detects immunoreactive and immunochemically nonreactive albumin has been introduced as an alternative to immunonephelometry. We investigated whether this technique is affected by sample temperature, particularly freezing.

Methods: Urine samples (n = 295) were collected from the general population (Prevention of Renal and Vascular End-Stage Disease Study). Samples were assessed by both immunonephelometry and HPLC when fresh and after storage at −20 °C for 4, 8, and 12 months and at −80 °C for 12 months.

Results: With immunonephelometry, storage for 4, 8, and 12 months at −20 °C resulted in mean (SD) urine albumin changes of −21% (29%), −28% (29%), and −34% (31) (P < 0.001 for trend). Storage at −80 °C resulted in a 5% (19%) change after 12 months of storage (not significant). With HPLC, storage for 4, 8, and 12 months at −20 °C resulted in urine albumin changes of −33% (28%), −43% (24%), and −55% (21%; P < 0.001 vs immunonephelometry). Storage at −80 °C resulted in a −29% (29%) change (P < 0.001 vs immunonephelometry).

Conclusion: Loss of albumin after freezing urine depends not only on freezing temperature but also on detection method. Detection of albumin by immunonephelometry appears to be significantly less influenced by freezing than detection by HPLC. Storage at −80 °C appears to prevent loss when using immunonephelometry, whereas HPLC still shows considerable loss even when urine is frozen at −80 °C. We propose that for reliable measurement of urine albumin, fresh samples should be used.

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Urine albumin measurements are used in patient care, epidemiological studies, and as intermediate endpoints in clinical trials. In the latter cases, urine samples are often stored frozen before assessment for practical reasons and for prevention of analytical day-to-day variation and drift. We recently showed that 1 year of frozen storage of urine at −20 °C results in an ~30% decrease in albumin concentrations, as well as an increase in the measurement error, in particular in samples with initial concentrations in the normoalbuminuric and microalbuminuric ranges (1, 2). Importantly, this change in urinary albumin concentrations (UACs) induced by frozen storage resulted in a significant decrease in predictive properties of urine albumin for mortality (2).

The decrease of albumin concentrations during frozen storage of urine has been attributed to several potential causes. One of these is the temperature at which frozen samples are stored. The effect of frozen storage has mainly been studied in urine samples stored at −20 °C; only a few studies have investigated frozen storage at lower temperatures. Results of 2 studies suggest that frozen storage at −70 °C is superior to storage at −20 °C (3, 4). Interpretation of these studies, however, is hampered by the fact that one study did not include fresh assessments (5) and the other did not find a significant difference between −70 °C and −20 °C, possibly owing to lack of power (3). A possibility that has not been studied to date is a potential effect of measurement technique. The current ways of measuring urine albumin are based on

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immunoreactive techniques. It is conceivable that prolonged freezing changes the immunoreactivity of the albumin molecule (thus producing an apparent decrease in albumin concentrations) (3, 6). Recently, a new method for assessment of urine albumin has been introduced. It detects immunochemically nonreactive albumin in addition to the immunoreactive albumin detected by conventional assays (7). It is not known whether and to what extent frozen storage affects urine albumin assessed by HPLC.

We compared the effect of prolonged frozen storage at −20 °C on urine albumin assessed by HPLC and an immunochemical assay (immunonephelometry) and investigated whether potential decreases in albumin could be prevented by storage at −80 °C.

Materials and Methods

Urine samples were collected from consecutive individuals visiting our outpatient clinic for the 3rd screening of the ongoing Prevention of Renal and Vascular End-Stage Disease (PREVEND) study (2004–2005) (8). Participants collected 24-h urine samples in plastic containers and stored the urine at 4 °C up to a maximum of 2 days before the visit to the outpatient clinic. For fresh determination of UAC by immunonephelometry and HPLC, samples were kept at 4 °C in polystyrene tubes for a maximum of 3 days. The maximum of refrigerated storage time for a fresh specimen would therefore be 5 days. Samples with a fresh concentration of immunoreactive albumin <5 mg/L were excluded to ensure potential detection of decrease with storage, given the lower detection limit of the assay of 2.4 mg/L. Samples with a fresh UAC of >200 mg/L were also excluded because the major differences between the 2 methods lie in the microalbuminuric and reference range. Portions of fresh urine were stored in polypropylene vials for assessment after storage for 4, 8, and 12 months at −20 °C (n = 295) and after 12 months at −80 °C in a subset of these samples (n = 224). All samples were subjected to hand-inversions and centrifuging before assessment.

Laboratory methods

We measured immunoreactive albumin by use of immunonephelometry (Dade Behring Diagnostics). The intraassay and between-day CVs were 2.7% and 4.5%, respectively. The variability rates for control and reagent lot changes were 1.1%–3.5% and 1.1%–2.1%, respectively. HPLC UAC was measured using an Agilent/Hewlett Packard 1100 HPLC with a limit of quantification of 3.0 mg/L. The intraassay and between-day CVs were 5.6% and 2.4%, respectively. Aliquots of urine (25 μL) were injected onto a Zorbax Bio series preparative GF 250 column (9.4 mm i.d. × 25 mm). The mobile phase was PBS (9 g/L NaCl, 0.775 g/L Na2HPO4, 0.165 g/L KH2PO4, pH 7.4) run at a flow of 0.5 mL/min. Peak integration was performed by use of the manufacturer-specified drop-line method (Accumin®, AusAm Biotechnologies).

We measured urine pH with Combur test strips (Roche), which have a measurement interval from pH 5 to 9; results are expressed as integers. We measured urinary creatinine by use of MEGA (Merck).

Statistical analyses

We performed statistical analyses by use of SPSS 14.0. Data are presented as mean (SD) percentage changes in albumin, which had a gaussian distribution. Skewed data were presented as median (interquartile range). We performed repeated measures analyses (MANOVA) to test differences between different time points for frozen storage at −20 °C. We performed correlation analyses according to Pearson, with log-transformation of variables with a skewed distribution. We analyzed differences between immunonephelometry and HPLC by use of paired-samples t-tests at the different time points and freezing conditions. Differences from zero were tested by 1-sample t-tests. A 2-sided P < 0.05 was considered to indicate statistical significance.

Results

We used urine samples of 295 individuals. Mean (SD) age was 55 (12) years and body mass index was 28 (4) kg/m²; 56% of the urine sample donors were males, and 5.9% had diabetes. Median (interquartile range) fresh urine albumin assessed by immunonephelometry was significantly lower than by HPLC [11 (7–51) mg/L vs 55 (34–111) mg/L, P < 0.001]. After 4 months of storage at −20 °C, the change in immunoreactive urine albumin was −21% (29%; P < 0.001 for difference with zero change). The change was −28% (29%) after 8 months and −34% (31%) after 12 months (P < 0.001 for trend; Fig. 1). When measuring urine albumin that was stored at −80 °C for 1 year, no change in albumin concentrations was observed [5% (19%), not significant]. Measurement of urine albumin by HPLC after frozen storage at −20 °C resulted in significantly higher changes (P < 0.001 for comparison of all time points with immunonephelometry) of −33% (28%) at 4 months (P < 0.001 for difference from zero change), −43% (24%) at 8 months, and −55% (21%) at 12 months (P < 0.001 for trend). Storage at −80 °C did not prevent the decrease [−29% (29%), P < 0.001].

Scatterplots of values before and after 12 months of storage are presented in Fig. 2. With storage at −20 °C and assessment by immunonephelometry (Fig. 2A), there was substantial variation in the stability of individual specimens, in particular at low albumin concentrations. Despite exclusion of samples with a fresh concentration <5 mg/L, and lower limit of detection 2.4 mg/L, a substantial amount of samples with low fresh concentrations presented with concentrations at or below the detection limit after frozen storage for 12 months. With storage at −80 °C and assessment by immunonephelomet-
metry (Fig. 2B), there were no decreases to below the
detection limit, and variation in stability was much less, in
particular in the lower range of concentrations. With
storage at −20°C and assessment by HPLC (Fig. 2C),
losses were more notable than with storage at −20°C
and assessment by immunonephelometry, in particular in
the higher range of concentrations. Variation in stability of
individual specimens was largest in the lower range of
concentrations. With storage at −80°C and assessment by
HPLC (Fig. 2D), losses were smaller over the whole range
of concentrations, but considerable variation in stability of
individual samples remained.

Given the large variation in stability between speci-
mens, we subsequently investigated whether percentage
change in concentrations after frozen storage is associated
with other factors, such as pH, urine creatinine con-
centrations, and fresh urine albumin concentrations. Urinary
pH was 5 in 38% of specimens, 6 in 44%, 7 in 15%, and 8
in 3%. The mean urine creatinine concentration was 9.6
(4.6) mmol/L. Respective correlations for pH, urine cre-
atinine, and log-transformed fresh albumin with percent-
age change after 12 months of storage at −20°C were $r =
0.26$ ($P <0.001$), $r = −0.22$ ($P = 0.001$), and $r = 0.33$ ($P
<0.001$) for nephelometry and $r = 0.13$ ($P = 0.05$), $r = 0.01$
($P = 0.90$), and $r = −0.05$ ($P = 0.44$) for HPLC. Respective
correlations with percentage change after 12 months of
storage at −80°C were $r = 0.22$ ($P = 0.001$), $r = 0.01$ ($P
= 0.86$), and $r = −0.16$ ($P = 0.02$) for nephelometry and $r =
0.05$ ($P = 0.42$), $r = −0.04$ ($P = 0.58$), and $r = −0.41$ ($P
<0.001$) for HPLC. Because decreases in urine albumin
concentrations are represented by negative percentages,
positive correlations indicate that with increasing values
of pH, urine creatinine, and log-transformed fresh albu-
mun, decreases become smaller, and vice versa for nega-
tive correlations.

Representative HPLC chromatograms before and after
storage up to 12 months at −20°C and for 12 months at
−80°C are shown in Fig. 3. One specimen with a fresh
HPLC albumin concentration of 31.9 mg/L decreased to
assessed concentrations of 9.9 mg/L, 8.3 mg/L, and
9.1 mg/L after 4, 8, and 12 months of storage at −20°C,
respectively (Fig. 3, A–D). After 12 months of frozen
storage at −80°C, the assessed concentration was 30.0
mg/L (Fig. 3E). For this specimen, the concentration
assessed by immunonephelometry was 5.6 mg/L. Results
for another specimen, with a fresh HPLC albumin
concentration of 55.0 mg/L, are shown in Fig. 3, F–J. For
this specimen, the concentration assessed by immuno-
nephelometry was 51.3 mg/L. For both specimens, fresh chro-
matographic patterns and those after storage at −80°C
for 12 months were very similar (Fig. 3, A vs E and F vs J).
Some extra fractions appeared after 4 months of storage in
the sample with a fresh HPLC concentration of 31.9 mg/L
(Fig. 3B), which became less prominent with longer frozen
storage (Fig. 3, C and D). Appearance of extra fractions
was less pronounced in the other specimen (Fig. 3G).

**Discussion**

We found greater susceptibility to the negative effects of
frozen storage on urine albumin assessed by HPLC than
by immunonephelometry. We also found that storage at
−80°C can prevent the decline that occurs with 1 year of
storage at −20°C if samples are assessed by immuno-
nephelometry, but not by HPLC. We also found con-
siderable variation in declines between samples and sig-
nificant correlations of these declines with urinary pH,
urine creatinine concentrations, and fresh albumin
concentrations.

To the best of our knowledge, our study is the first to
investigate the effect of prolonged frozen storage on urine
albumin assessed by HPLC. One study that investigated a
maximum of 2 months of storage reported no change in
measured albumin concentrations (7). Neither data nor
baseline albumin were presented, however, so the power
of that study for detecting changes cannot be estimated, in
particular because the change in albumin concentration
associated with freezing dependent on time is highly
variable between individuals and dependent on the initial
concentrations (1, 2). Our study shows that frozen storage for at least 4 months and beyond has a profound effect on UACs assessed by HPLC.

The results of our study are relevant not only to the interpretation of individual studies that use frozen samples of urine for albumin measurement, but also to the interpretation of previous studies that compare urine albumin assessed by HPLC with immunochemical methods (6, 7, 9–15). All studies to date used urine samples that had been stored for varying durations of time at −20 °C or −70 °C/−80 °C before assessment of urine albumin by HPLC. In some of these studies, immunoreactive albumin was assessed from fresh samples (9, 12, 16, 17), whereas others used frozen samples (6, 11, 13, 14). In a previous study in which we compared nephelometric values from fresh samples with HPLC values from frozen samples, we found for the normoalbuminuric range that urine albumin assessed by HPLC is a factor of 3 higher than urine albumin assessed by immunonephelometry, and a factor of 1.3 higher in the microalbuminuric range (9). In the present study, we found a factor of 5 difference between HPLC and immunonephelometry over this concentration range as a whole if both assessments were performed in fresh samples. Relevant
comparisons can therefore be made only if both assays have been performed in fresh samples. Because of the greater effect of frozen storage, it is likely that use of HPLC measurement of frozen samples will lead to more underestimation (compared to classic methods) both in terms of prevalence of microalbuminuria and as a predictor of events.

We found positive correlations between urinary pH and percentage change in albumin concentrations after 12 months of storage at −20 °C and after 12 months of storage at −80 °C. These correlations indicate smaller decreases at higher values of pH. The mechanism underlying this observation may involve increased aggregation and/or denaturation of albumin at relatively low pH because of the isoelectric point of albumin at pH 4.7. The 1st of the 2 series of representative HPLC chromatograms showed appearance of early peaks and disappearance of the albumin peak after 4 months of storage at −20 °C (Fig. 3B). This result is likely to be the consequence of aggregation of albumin molecules and possibly of other proteins. The later disappearance of these peaks after 8 and 12 months of storage could be consistent with degradation. We also found a significant inverse correlation between urine creatinine concentrations and percentage change in

Fig. 3. Representative HPLC chromatograms of 2 albumin samples assessed before and after 4, 8, and 12 months of storage at −20 °C and after 12 months of storage at −80 °C.

(A), example 1: UAC (31.9 mg/L) fresh urine. (B), example 1: UAC (9.9 mg/L) after 4 months of storage at −20 °C. (C), example 1: UAC (8.3 mg/L) after 8 months of storage at −20 °C. (D), example 1: UAC (9.1 mg/L) after 12 months of storage at −20 °C. (E), example 1: UAC (30.0 mg/L) after 12 months of storage at −80 °C. (F), example 2: UAC (55.0 mg/L) fresh urine. (G), example 2: UAC (11.3 mg/L) after 4 months of storage at −20 °C. (H), example 2: UAC (6.7 mg/L) after 8 months of storage at −20 °C. (I), example 2: UAC (5.2 mg/L) after 12 months of storage at −20 °C. (J), example 2: UAC (48.3 mg/L) after 12 months of storage at −80 °C. Arrows in the Fig. indicate urinary albumin peaks.
albumin concentration after 12 months of storage, but only for storage at −20 °C and assessment by immunonephelometry. These findings are consistent with greater decreases in concentrations in more concentrated urine samples. Correlations of percentage changes in albumin concentrations with fresh albumin concentrations are consistent with relatively small percentage decreases in samples with high fresh albumin concentrations for assessment by immunonephelometry and storage at −20 °C, whereas opposite correlations were present for assessment by immunonephelometry and storage at −80 °C and for assessment by HPLC and storage at −80 °C. The opposite correlations for assessment by immunonephelometry between storage at −20 °C and −80 °C may be explained by the fact that aggregation is prevented to a large extent with storage at −80 °C for almost all samples, with the significant correlation for the greater part being the consequence of some samples in which decreases in assessed albumin concentrations occurred.

For this study, we used the routine HPLC commercially available method (Accumin), which makes use of drop-line integration. Recently, the accuracy of the HPLC assay and the integration method has been debated. Sviridov et al. (18) showed by use of mass spectrometry that the size exclusion–based HPLC method is incapable of completely resolving the albumin molecule from other proteins in the urine. These authors found that the peak indicated as albumin contained up to 20% other proteins such as transferrin, α1-proteinase inhibitor, and α1-acid glycoprotein. Further studies will be needed to investigate the content of the peak and the outcome of potential differences in integration methods such as through-to-through basis.

We did not further study the reason for the greater sensitivity of HPLC measurement to the effects of prolonged freezing. One potential explanation might be that the additional albumin molecules that are measured by HPLC are more susceptible to loss with frozen storage. As far as the effect of different urine freezing temperatures on albumin changes, correct interpretation of previous studies is hampered by lack of fresh assessments in more concentrated urine samples. Correlations of percentage changes in albumin concentrations with fresh albumin concentrations are consistent with relatively small percentage decreases in samples with high fresh albumin concentrations for assessment by immunonephelometry and storage at −20 °C, whereas opposite correlations were present for assessment by immunonephelometry and storage at −80 °C and for assessment by HPLC and storage at −80 °C. The opposite correlations for assessment by immunonephelometry between storage at −20 °C and −80 °C may be explained by the fact that aggregation is prevented to a large extent with storage at −80 °C for almost all samples, with the significant correlation for the greater part being the consequence of some samples in which decreases in assessed albumin concentrations occurred.

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In conclusion, the decrease of albumin concentrations after freezing urine for a prolonged time period depends not only on the freezing temperature but also on the detection method. Detection of albumin by immunonephelometry appears to be significantly less influenced by freezing than by HPLC. Storage at −80 °C appears to prevent loss when using immunonephelometry, whereas HPLC detection still shows considerable loss even when urine is frozen at −80 °C. We propose that for reliable measurement of urine albumin, fresh samples should be used, particularly in the normo- and microalbuminuric range. Previous urine albumin data (and studies) obtained with frozen samples should be interpreted with extreme caution.

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