Falsely Low Urinary Albumin Concentrations after Prolonged Frozen Storage of Urine Samples, Jacoline W. Brinkman,1 Dick de Zeeuw,1 Jacko J. Duker,1 Ronald T. Gansvoort,2 Ido P. Kema,3 Hans L. Hilleges,4 Paul E. de Jong,2 and Stephan J.L. Bakker2* (Departments of 1 Clinical Pharmacology, 2 Internal Medicine, and 3 Pathology and Laboratory Medicine, and the 4 Trial and Coordination Center, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands; * address correspondence to this author at: University of Groningen and University Medical Center Groningen, Department of Internal Medicine, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands; fax 31-50-3639069, e-mail s.j.l.bakker@int.umcg.nl)

Microalbuminuria, defined as a urinary albumin concentration (UAC) of 20–200 mg/L, is an early predictor of diabetic nephropathy (1–6). In addition, microalbuminuria is a marker of cardiovascular morbidity and mortality, both in patients with diabetes mellitus and in the general population (7–17). Consequently, there is great interest in screening for microalbuminuria in these groups. In cohort studies, urine samples are often kept frozen at −20 °C before analysis. Although some study results have indicated no effect of freezing on UAC (18–23), other studies have found erroneously low values when samples were frozen at −20 °C (24–27). Only a few studies have investigated the effect of longer storage periods, but these studies were small, and samples were in the macroalbuminuric range (>200 mg/L).

We investigated the effects of storage at −20 °C for up to 24 months, mixing methods, and baseline UAC on samples in the normo- and microalbuminuric ranges.

Urine samples were collected during the prospective PREVEND study in the general population initiated to investigate urinary albumin excretion as a predictor of renal and cardiovascular disease (28). The participants were asked to collect urine for two 24-h periods and to store it in two 3-L plastic containers at 7 °C and deliver it within 2 days to the clinic. Immediately after delivery, the urine sample volume was determined, and a portion of each of the 24-h samples was stored in 2-mL polypropylene aliquots at −20 °C for albumin assessment after freezing. For measurement of UAC in fresh samples, portions were kept at 7 °C in 10-mL polystyrene tubes until the UAC was measured.

All participants gave written informed consent. The PREVEND study was approved by the local medical ethics committee and was conducted in accordance with the guidelines of the Declaration of Helsinki.

On the basis of the fresh UAC and duration of storage, we selected 1785 urine samples for the study. Samples were obtained from storage (−20 °C) 1–3 days before analysis, thawed at 7 °C, and randomly assigned to 3 groups. Samples in the first group (I; n = 600) were not mixed before analysis, samples in the second group (II; n = 596) were subjected to 3–4 hand inversions before analysis, and samples in the third group (III; n = 689) were vortex-mixed for 5–10 s. All samples were centrifuged before analysis and analyzed directly after centrifugation. At the time of analysis the samples were at room temperature. Samples were thawed and analyzed in the same laboratory.

UAC was measured by immunonephelometry (Dade Behring Diagnostics) with a lower limit of detection of 2.3 mg/L (defined as the concentration of the lowest calibrator solution). The intra- and interassay CVs, evaluated in our laboratory, were 2.7% and 4.5%, respectively.

All data were analyzed with SPSS 12.0 software. Data are presented as mean (SD) percentage differences in albumin, which had a gaussian distribution. Differences among groups were assessed by ANOVA, and differences between groups by post hoc analysis according to Tukey. t-Tests for single groups were used to test whether differences in UAC were statistically different from zero.

To investigate whether the effect of duration of storage and baseline UAC independent of UAC difference, we performed a multiple linear regression analysis using percentage UAC difference as an independent variable. Potential interactions were tested. Statistical significance was determined as a P value <0.05.

After 3–5 months of storage, there was a considerably larger change in UAC in unmixed samples than in samples that were subjected to either vortex-mixing or hand inversions [−34.9 (28.6)% vs −5.3 (30.6)% and −2.6 (27.5)%, respectively; P <0.001]. These latter two changes were not markedly different from zero. Only the samples of groups II and III were included in further analyses. The differences amounted to −23.7 (26.2)% and −26.1 (24.7)% after 18–24 months of storage, respectively (P <0.001 in both groups). Overall post hoc analysis showed no major differences in albumin decrease between groups II and III (P >0.05) for all durations of storage and concentration categories; therefore, for further analysis, we combined the 2 groups. The combined data for percentage change according to concentration categories and duration of storage are shown in Table 1. The UAC difference stabilized at −30% after 8 months of storage. The large SD in the changes in UAC suggests large differences among samples in the response of UAC to freezing.

The percentage changes in albumin concentration in individual samples after 3–5 months or 18–24 months are shown in Fig. 1. Specimens with a high baseline UAC responded differently to freezing than those with a low UAC. A maximum change of −28 (33)% was seen in the 10–20 mg/L category. The samples in the concentration group 200–500 mg/L showed a markedly smaller change compared with the samples in the other groups (P <0.001). Multiple regression analysis showed no interaction between initial UAC and duration of storage.

The data indicate that urine samples can be stored for 5 months without great changes in mean albumin concentration when samples are mixed adequately after thawing. The change in UAC varies among samples with large variations in the lower concentration ranges.
Although several studies have investigated the effect of freezing on UAC, most studied only shorter storage times, different concentrations of albumin, and smaller numbers of urine samples. Innanen et al. (21) and Collins et al. (22) found no substantial difference after 6 months. Two authors reported no substantial change in UAC after 24 months (29) and 26 months (30); the UACs in those studies, however, were considerably higher than in ours. In the first study (29), a nonsignificant 10% loss in albumin was seen in 10 samples, a change consistent with the ~10% decrease we found in samples with a concentration of 200–500 mg/L. Shield et al. (30) also reported that urine samples with higher albumin concentrations are less prone to change in UAC after freezing for up to 6 months.

We emphasize the importance of the effect of freezing on samples in the microalbuminuric (20–200 mg/L) range because microalbuminuria is an early predictor of cardiovascular and renal disease (7, 14, 16, 17).

Why UAC decreases during freezing is still unknown. It may be that albumin molecules are trapped in the precipitate because of an alteration in pH of the urine samples during freezing and thawing (20, 31). Bacterial contamination may also affect UAC. Falsely low values may reflect hydrolysis by bacterial proteases (32). Moreover, storage tube materials might play a role in preventing albumin loss after freezing. Collins et al. (22), however, found no difference in concentration when they studied the effect of different tube materials on UAC after freezing. Conformational changes that occur during freezing may lead to loss of the antibody recognition site that is needed to measure albumin with immunochemical methods (22, 32).

Our study is limited by the fact that we did not investigate possible mechanisms of loss of UAC or the effect of storage at −70 °C, at which temperature MacNeil et al. (26) found no substantial decrease in UAC after 160 days of storage. We found a large variability in UAC change, a common phenomenon in other studies as well (18, 21, 24, 25, 33, 34). A thorough investigation of all the circumstances that might be involved in the change in albumin after freezing was beyond the scope of our study.

We recommend that albumin be measured in fresh urine samples whenever possible—because specimens respond differently to freezing and thawing and because albumin appears to be less stable in normo- and microalbuminuric samples than in samples with higher albumin concentrations. This effect of freezing must be taken into consideration when microalbuminuria is used as a risk predictor.

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Use of Fully Denaturing HPLC for UGT1A1 Genotyping in Gilbert Syndrome, James R. Harraway* and Peter M. George (Molecular Pathology Laboratory, Canterbury Health Laboratories, Christchurch, New Zealand; * ad-
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Gilbert syndrome is an inborn error of bilirubin conjugation
caused by mutations in the conjugating enzyme
uridine diphosphate glucuronosyltransferase (UGT1A1) (1).
UGT1A1 activity is moderately reduced in Gilbert syn-
drome, causing low-grade nonhemolytic unconjugated
hyperbilirubinemia without other biochemical or clinical
features of hepatic or hematologic pathology. Bilirubin
concentrations typically fluctuate over time and tend to be
increased in response to metabolic stress such as intercur-
rent illness and fasting (2).

The clinical importance of Gilbert syndrome derives from
its high population prevalence. The causative mutation
in Caucasians is almost exclusively a dinucleotide
insertion in the TATA box of the UGT1A1 promoter. The
most common UGT1A1 promoter contains the sequence
(TA)6TAA; insertion of an extra TA reduces expression
of the UGT1A1 gene to 20%–30% of reference values (3).
The frequency of the (TA)6TAA allele (also known as
UGT1A1*28) in the Caucasian population is ~0.35; there-
fore, nearly all Caucasians with Gilbert syndrome are
homozygous for this allele (4). UGT1A1*28 is less frequent
in Asians. A significant proportion of Asians with Gilbert