Effects of Storage Time and Temperature on Measurement of Small Concentrations of Albumin in Urine

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Accurate measurement of albumin excretion rates is important in choosing treatment regimens that may reverse early diabetic renal damage. We report here determinations of slight albuminuria ("microalbuminuria") by radioimmunoassay of fresh specimens, frozen aliquots (stored at -20 °C for two, eight, and 24 weeks), and refrigerated specimens (stored at 4 °C for one, two, and eight weeks). Seven separate analyses were performed on 101 specimens of urine obtained from 37 subjects with insulin-dependent diabetes mellitus and from 10 nondiabetic healthy controls of a similar age. Storage of urine samples at -20 °C resulted in significantly lower measurements of microalbuminuria than in fresh urine (ANOVA, corrected for repeated measures: P = 0.01 to 0.0001). In contrast, storage of urine samples at 4 °C for as long as eight weeks did not significantly affect urinary albumin results. The pH values of the specimens were minimally altered and were not a likely cause of the decreased albumin values in the frozen specimens. We conclude that urine specimens for microalbuminuria measurements should either be analyzed as fresh specimens or stored at 4 °C and assayed as soon as possible.

Additional Keyphrases: albumin excretion rate · diabetes mellitus · radioimmunoassay · sample handling · variation, source of

An increased albumin excretion rate (AER) measured overnight or during resting reflects an incipient stage of diabetic nephropathy in the absence of urinary tract infection (1-4). Accurate measurements of urinary albumin excretion are important clinically, because of speculation that early diabetic renal damage may be reversible if albumin spillage can be detected while still slight ("microalbuminuria"). The range of AER within which reversibility with medications is considered possible currently is believed to be between 30 and 200 μg/min (5, 6). Studies in progress at our Center suggest that spillage in the 10 to 30 μg/min range in overnight urine collections may be reversible just by improved glucose control. Similarly, exercise-induced microalbuminuria, which may be present in subjects who still have a normal overnight AER of <10 μg/min, may be reversible with improved glucose control (7).

It is imperative to have accurate determinations of small amounts of albumin spillage. Radioimmunoassay is capable of yielding accurate laboratory determinations. However, more information is needed about the effect of specimen storage conditions on the accuracy of results. A recent study (8) showed that storage of urine specimens for two or six months at -20 °C resulted in falsely low albumin concentrations measured nephelometrically. The purpose of the present investigation was to study, with use of a standard radioimmunoassay, the effects of storage at various temperatures and for various periods on measurements of albumin in urine.

Materials and Methods

Thirty-seven subjects with Type I diabetes and 10 nondiabetic controls of similar age provided 101 specimens of fresh urine for albumin determinations. Twenty-five of the specimens from subjects with diabetes and eight of the specimens from nondiabetic controls were collected after the subjects had ridden a standard exercise bicycle for 20 min. Fifty-eight of the specimens from subjects with diabetes and 10 of the specimens from nondiabetic controls were timed overnight urine collections.

The volume, time, and duration of each specimen were noted, and six well-mixed aliquots of each specimen were taken and stored at (a) 4 °C for one, two, and eight weeks or (b) -20 °C for two, eight, and 24 weeks. All albumin determinations were performed in duplicate. The albumin concentration in a seventh (fresh) aliquot of each specimen was assayed on the day of collection.

For each aliquot, we also determined pH (with a Model 40 pH meter; Beckman Instruments Inc., Brea, CA 92621) and gross protein content (with Albustix®; Ames Division, Miles Laboratories, Inc., Elkhart, IN 46515) at the time of the albumin assay. The Albustix results were used to help decide whether to dilute each sample, so that the albumin concentration could be determined in the most sensitive portion of the standard curve. We have included the results of the Albustix screening to highlight the variability in urinary measurements of albumin in comparison with quantitative RIA determinations. Unfortunately, in many physicians' offices, Albustix is still the only screening test used.

At the appropriate time, we let each refrigerated or frozen aliquot come to ambient temperature and then mixed it thoroughly. All samples were centrifuged, to clear them of particulates, before analysis for albumin.

Microalbuminuria was determined by using a radioimmunoassay of albumin (Albumin Double- Antibody Kit; Diagnostic Products Corp., Los Angeles, CA). The accuracy at the lower detection limit was improved by routinely including a 2.5 mg/L albumin standard in the standard curve. The AER detection limit of this method in our laboratory is 0.5 μg/min; our intra-assay and interassay variations (CVs) are 1.7%–4.4% and 3.6%–7.4%, respectively.
Depending on the concentration of albumin in the fresh urine, we divided the specimens into five groups: (a) 45 samples for which the albumin concentration in fresh urine (expressed as AER) was \( <7.6 \, \mu g/min \); (b) 25 samples with albumin concentrations from 7.6 to 18.0 \( \mu g/min \); (c) 10 samples with 18.1 to 30.0 \( \mu g/min \); (d) 12 samples with 30.1 to 200 \( \mu g/min \); (e) nine samples with gross microalbuminuria, \( >201 \, \mu g/min \). These divisions were selected arbitrarily, to determine whether any particular concentration of albumin might be more affected by the time and temperature of storage than another.

**Statistical methods.** The albumin results from fresh urine specimens and from specimens stored for various periods and in various temperature conditions were analyzed by using the SAS program (9). Statistical analyses included use of the chi-square test, paired and two-independent-sample Student’s t-tests, correlation coefficient, and repeated measure analysis of variance (ANOVA) corrected for repeated measures. Multivariate and univariate analyses were used to determine global differences. For individual statistical analyses we used Bonferroni’s method (10).

**Results**

The mean age of the 37 subjects with insulin-dependent (Type I) diabetes mellitus was 20.2 years; that of the 10 nondiabetic controls was 24.2 years (\( P > 0.05 \); chi-square test). There were five males and five females in the control group, and 23 males and 14 females among the subjects with diabetes. The mean albumin values for the fresh urine specimens and for specimens after storage at 4 °C for one, two, or eight weeks or after storage at \(-20 \, °C\) for two, eight, or 24 weeks are shown in Table 1. The values for all stored specimens were significantly different from results for fresh specimens by global multivariate analysis (\( P = 0.0008 \)). The albumin concentrations in fresh urine were significantly different from the concentrations in aliquots from the same urine specimens kept at \(-20 \, °C\) for two (\( P = 0.01 \), or eight and 24 (\( P = 0.0001 \)) weeks (ANOVA corrected for repeated measures). The differences at eight and 24 weeks for the specimens stored at \(-20 \, °C\) reflected the specimens with concentrations \( \leq 30 \, \mu g/min \) (multiple Student's t-tests, with Bonferroni's correction for number of comparisons). By contrast, albumin concentrations in fresh urine specimens did not change significantly after storage at 4 °C for one, two, or eight weeks. At high amounts of albumin excreted (\( >30 \, \mu g/min \)), there were no significant differences for any of the storage time periods or temperature studies compared with the fresh urine specimens (multiple t-tests, with Bonferroni's correction).

The alterations in albumin results were similar in urine specimens from subjects with diabetes compared with those from nondiabetic controls. Likewise, there was no difference if the specimen was an overnight collection or was obtained post-exercise.

The mean (±SEM) pH of 101 samples in the fresh aliquot was 5.80 ± 0.06. The maximum change in specimens frozen at \(-20 \, °C\) for up to 24 weeks was 5.88 ± 0.006. Specimens stored at \(-20 \, °C\) for two, eight, and 24 weeks had significantly higher pH values than did the fresh aliquot (\( P < 0.05 \); ANOVA with Bonferroni's correction for repeated measures). However, the changes (±0.08 units) were not considered clinically important. The maximum change for specimens kept at 4 °C for up to eight weeks was 5.88 ± 0.07.

The logarithm of albumin concentrations for fresh and all other specimens correlated with the Albustix readings (\( r = 0.74 \) to 0.83; \( P < 0.0001 \)). Figure 1 shows the correlation in log scale of Albustix readings with mean ± SEM AER (\( \mu g/min \)) for the fresh specimens. However, Albustix results were not useful as individual quantitative values. The Albustix was negative for 77 specimens for which the mean (±SEM) AER was 9.9 ± 1.0 \( \mu g/min \); for three of these specimens the AER was >30 \( \mu g/min \). The Albustix gave a "trace" reading for 14 specimens; the mean AER for these specimens was 75.1 ± 26.6 (range 2.4 to 389 \( \mu g/min \)). Five specimens gave a "1+" reading and had a mean AER of 402.2 ± 156.3 \( \mu g/min \). Five other specimens gave a "2+" reading and had a mean AER of 1177.4 ± 552.8 \( \mu g/min \).

**Discussion**

Using a sensitive radioimmunoassay to measure albumin concentrations in urine, we compared results from fresh urine specimens with results after storage for various times and at different temperatures. We found significantly lower concentrations of albumin in urines frozen at \(-20 \, °C\) for two, eight, and 24 weeks. There was no deterioration of albumin concentration by eight weeks if the urine had been stored at 4 °C.

Freezing primarily affected the storage of specimens with initial albumin contents (AER) \( \leq 30 \, \mu g/min \). This false decrease could lead to classifying the albumin content as normal rather than "borderline-elevated" and overlook an important stage of albumin spillage during which improved glucose control alone might reverse the process.

The changes in pH were not considered clinically significant. These pH changes also were present in the specimens stored at 4 °C for two weeks, in which changes in albumin concentrations were not observed. It is unlikely that the changes in pH were responsible for the reduced albumin concentrations of the frozen specimens. Elving et al. (8) previously noted "no relation between pH and precipitate formation." In contrast, Townsend et al. (11, 12) showed that adjustment of urine pH to neutral before or after deep-freeze storage prevented the decrease of albumin.

| Table 1. Effect of Temperature and Duration of Storage on AER (Mean ± SEM), Measured in 101 Urine Specimens |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| AER in fresh urines, \( \mu g/min \) | Ambient, 0      | 4 °C, 1         | 4 °C, 2         | \(-20 \, °C, 2^*\) | 4 °C, 8         | \(-20 \, °C, 8^*\) | \(-20 \, °C, 24^*\) |
| <7.6 (45)*                        | 4.4 ± 0.3       | 4.5 ± 0.3       | 4.3 ± 0.3       | 3.6 ± 0.3       | 4.6 ± 0.3       | 2.8 ± 0.3       | 2.3 ± 0.3       |
| 7.6-18.0 (25)                     | 12.0 ± 0.7      | 11.7 ± 0.9      | 11.8 ± 0.8      | 10.4 ± 0.9      | 11.7 ± 1.0      | 8.8 ± 1.0       | 7.9 ± 0.9       |
| 18.1-30 (10)                      | 22.8 ± 1.0      | 20.3 ± 0.7      | 20.6 ± 0.8      | 18.9 ± 1.2      | 19.1 ± 0.9      | 15.4 ± 1.8      | 13.7 ± 2.0      |
| 30.1-200 (12)                     | 78.1 ± 11.3     | 86.3 ± 13.7     | 87.5 ± 14.4     | 77.3 ± 14.0     | 81.8 ± 13.6     | 75.6 ± 15.5     | 78.8 ± 14.8     |
| >201 (9)                          | 894.8 ± 317.5   | 774.3 ± 261.6   | 837.0 ± 300.8   | 831.6 ± 299.2   | 812.2 ± 282.3   | 728.2 ± 240.2   | 831.3 ± 282.1   |

* The number of specimens analyzed in each group is shown in parentheses. Temperature and duration (weeks) of storage. Results significantly different from fresh samples (using ANOVA corrected for repeated measures): \( P = 0.01 \) at two weeks; \( P = 0.001 \) after eight and 24 weeks.}

CLINICAL CHEMISTRY, Vol. 36, No. 8, 1990 1429
concentrations. This effect was considered to apply only to specimens centrifuged before analysis and not to those vortex-mixed (11, 13). However, Silver et al. (14) did not find significant differences between vortex-mixed and centrifuged specimens, and we did not evaluate differences between vortex-mixed and centrifuged specimens.

We grouped the results of the albumin concentrations (Table 1) related to clinically relevant AER values. We previously reported the value of 7.6 μg/min as the upper limit of normal for overnight albumin spillage in normal controls (7). We consider 18.0 μg/min as the upper limit of normal for timed daytime collections, higher than for the overnight specimens because of the influences of exercise on albuminuria. Values >30 μg/min are associated with a 95% likelihood of progressing to diabetic nephropathy (4). Finally, we included the AER value of 200 μg/min because this is the upper limit at which incipient nephropathy currently is considered reversible by medications.

Freezing urine specimens may cause conformational change in urinary proteins, resulting in a partial precipitation. Ermann et al. (15) studied the effect of freezing on albumin concentrations; using human albumin antibodies purchased from Biocides (Rehovot, Israel) they found that freezing decreased the results by ~20%. Elving et al. (8) measured urinary albumin concentrations with a nephelometer and also noted reductions in albumin concentrations after storing the specimens at −20 °C for two or six months. Our present report is, to our knowledge, the first evaluation of the effects of freezing on albumin concentrations in urine as determined with a commonly available commercial radioimmunoassay kit.

We are frequently told by practitioners that they screen their patients' urines at clinic visits with Albustix and that a more sensitive test is not needed. However, as the present data show, individual Albustix values vary tremendously for a given albumin concentration. Defining specific cutoff values at which incipient diabetic nephropathy can be detected and reversed is possible only by using an accurate quantitative test such as the radioimmunoassay for albumin.

We conclude that frozen urine specimens should not be used for albumin determinations, because freezing may lower the values. This underestimation limits the ability to diagnose borderline albuminuria. We recommend measuring albumin either in fresh urine specimens or in urine stored at 4 °C and assayed within eight weeks of collection.

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