Screening for Microalbuminuria in Patients with Diabetes Mellitus: Frozen Storage of Urine Samples Decreases Their Albumin Content


The influence of storage on urinary albumin concentration was prospectively studied with use of overnight urine specimens (Albustix negative) from 73 diabetic patients. From each urine sample four aliquots were taken. One was stored at 4 °C and assayed within two weeks, the other three were stored at −20 °C and assayed within two weeks and after two and six months. Albumin concentration was measured with laser immunonephelometry. The detection limit, 1 mg/L, suffices for the screening of diabetic patients for microalbuminuria. After storage for two and six months at −20 °C, significantly lower albumin concentrations were found. The difference was mainly caused by lower concentrations found in urine samples in which a precipitate had formed, which was the case in 22 and 25 samples, respectively. Thus, freezing of urine samples for determination of low concentrations of albumin may yield falsely low results. Urine samples are best stored at 4 °C and assayed within two weeks.

Additional Keyphrases: diabetic nephropathy · laser immunonephelometry

About 30% to 40% of all patients with type 1 (insulin-dependent) diabetes mellitus will develop the clinical syndrome of diabetic nephropathy, characterized by persistent proteinuria (≥0.3 g/24 h), hypertension, and deterioration of renal function. It invariably leads to end-stage renal failure (1). Manifest diabetic nephropathy is preceded by a phase in which albuminuria is abnormally increased but not yet detectable with routine methods. This microalbuminuria (20–200 μg/min, i.e., 30–300 mg/24 h) is highly predictive of future development of manifest diabetic nephropathy (2). In patients with microalbuminuria, strict metabolic control can retard the progression toward manifest diabetic nephropathy (3). Screening of diabetic patients for microalbuminuria therefore has become part of routine diabetes care. This screening concerns large groups of patients, so a simple, sensitive, and inexpensive method should be available for measuring low concentrations of urinary albumin. Here we describe a laser immunonephelometric method that meets these requirements.

Little is known about the effect of storage on urinary albumin concentrations. Urine samples often are stored at −20 °C for long periods before being analyzed. Thus we have performed a prospective study to evaluate the effects of freezing urine samples for longer periods on measured albumin concentration.

Materials and Methods

Samples. Overnight urine specimens were collected from 73 nonproteinuric (Albustix negative) diabetic patients, consecutively attending the outpatient diabetes clinic. The specimens were centrifuged (1500 × g, 15 min) and the supernate was divided into four 2-mL aliquots and stored at 4 °C for determination within two weeks and at −20 °C for determination within two weeks and after two and six months, respectively. Before assay, all samples were examined for the presence of a precipitate and again centrifuged (1500 × g, 15 min). In 33 urine samples the albumin concentration was also measured within two days after collection. The pH of 59 urine samples was measured.

Apparatus. Urinary albumin concentrations were measured with the Disc 120 Nephelometer (Hyland, Nivelles, Belgium).

Standards and controls. A pool of serum from 500 donors was stored at 4 °C for two weeks. It then was centrifuged (60 000 × g, 1 h) and precipitates and lipids were removed. The supernate was filtered consecutively through 0.45-μm and 0.22-μm pore-size filters (Millipore Corp., Bedford, MA), quick-frozen in 0.7-mL portions by use of a solid CO2-ace-tone mixture, and stored in sealed ampules at −70 °C. Two control urines, one with high and one with low albumin concentration, were prepared and stored in an identical way.

Antiserum. Antiserum was raised in New Zealand White rabbits as follows. For immunization we used 0.5 mg of human serum albumin (Behringwerke, Frankfurt, F.R.G.) and complete Freund’s adjuvant. After three and six weeks, booster injections were given, consisting of 0.5 mg of albumin in incomplete Freund’s adjuvant. When the optimal antibody titer was reached, the rabbit was bled. Antibody specificity was checked by Ouchterlony double immunodif-fusion and by immunoelectrophoresis. Titer and avidity were estimated by laser immunonephelometry. The serum, stored at −20 °C, was diluted 300-fold in diluent (see below) immediately before use. This dilution factor was chosen to get high sensitivity without getting into the range of antigen excess. After 1 h at room temperature the diluted antiserum was filtered through a 0.22-μm filter to minimize antiserum blanks.

Diluent. The diluent was phosphate-buffered isotonic saline (phosphate 5 mmol/L, pH 7.4) containing, per liter, 20 g of polyethylene glycol 4000 (Merck, München, F.R.G.) and 20 g of polyethylene glycol 2000 (Merck), plus 0.25 g of NaN3 as preservative. This diluent gave low light scatter in blanks in combination with high sensitivity. Before use it was filtered through 0.22-μm filters. It is stable at room temperature.

Assay procedure. The standard was first diluted 100- and 1000-fold with isotonic saline. Subsequently, dilutions from five- to 80-fold were made to get six-point standard curves. The concentrations were 5.5, 11, 22, 44, 65, and 87 mg/L for the high range and 0.6, 1.1, 2.2, 4.4, 6.5, and 8.7 mg/L for the low range. At the beginning and end of each run, both sets of standards were assayed. For the high range, the signal was measured at gain 11; for the low range, at gain 12. The detection limit was 1.0 mg/L. All samples were first tested with Albustix (Ames Miles, Paris, France). For samples with high concentrations of albumin, 10 μL of undiluted or
of twofold diluted urine was mixed with 1000 μL of diluted antiserum (or diluent); and for the lower concentrations a 100-μL urine sample was used. Incubation was for exactly 1 h. The relative light scatter from all samples was then measured nephelometrically.

Calibration and standard serum. The standard pool was calibrated nephelometrically for albumin vs purified serum albumin (Behringwerke). There was consistent parallelism between the six-point calibration curves, the standard pool, and the urine samples, respectively.

Statistical analysis. Results are presented as mean and standard deviation (SD), or median and range. Albumin concentrations in samples stored for two weeks or for two or six months at −20 °C were compared with those in samples stored for two weeks at 4 °C by use of Wilcoxon’s signed rank test for paired data. We also applied Bonferroni’s adjustment for multiple comparisons.

Results

Analytical Variables

Precision. Albumin in urine samples was determined in duplicate. Table 1 shows the between-run variation, measured over a one-year period, for the high- and the low-concentration control urine samples. Both samples remained free of precipitate. The intra-run CV was 1.8% for two urine samples, one in the high and one in the low range.

Analytical recovery. Recovery was determined after adding diluted standard serum to urine samples containing 10–22 or 1.5–5.6 mg of albumin per liter. Albumin concentrations were measured before and after addition of the standard serum (albumin content 20.6 or 41.2 mg/L, or 2.2 or 4.4 mg/L, respectively). The mean (and SD) recovery was 96.7% (3.9%) (n = 8) and 103.2% (9.0%) (n = 7) for the high and low ranges, respectively.

Effects of Storage Conditions

Thirty-three urine samples, stored at 4 °C, were analyzed for albumin concentration within two days and within two weeks after collection. No significant difference in albumin concentration was found (14.1 ± 20.4 vs 14.3 ± 19.9 mg/L). Subsequently, after different periods of storage, we tested for albumin concentration in urine samples from 73 diabetic patients without proteinuria (Albustix negative), using the values for samples stored at 4 °C for two weeks as the reference value. The mean urinary albumin concentration of these 73 samples, stored at 4 °C for two weeks, was 17.9 ± 28.4 mg/L. Storage for two weeks at 20 °C produced no significant change in results, although the albumin concentration was clearly decreased in two samples. After two and six months of storage at −20 °C, significant differences in albumin content were evident (Table 2).

As shown in Figure 1, the albumin concentration decreased, especially in samples in which a precipitate had formed during storage. After two and six months, a precipitate had formed in 22 and 25 of the urines, respectively. The pH was not measured in all urines, but the mean pH of 20 urines with a precipitate and of 39 urines without precipitate was not significantly different: 5.7 ± 0.5 and 6.0 ± 0.6, respectively. Microscopic examination of the precipitate showed that it consisted almost entirely of urates, both amorphous and crystalline.

Discussion

In this study we used a rapid, sensitive method for measuring low concentrations of albumin, a modification of a method previously described (4). The intra- and interassay

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<td>2 months, −20 °C</td>
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Significantly different from results after <2 weeks at 4 °C: * P < 0.03; b P < 0.005.

Table 2. Albumin Concentrations in Urine Samples after Storage for Various Periods

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CVs for the laser immunonephelometry—2% and 10%, respectively—compare well with those for techniques such as radioimmunoassay, enzyme-linked immunoassay, radial immunodiffusion, and immunoniturbidimetry (5–7). The detection limit of 1 mg/L suffices for the screening of diabetic patients for microalbuminuria. Automation of the method facilitated analysis of large runs of samples. Smaller runs, however, can also be handled easily and do not make the method more expensive. The low cost of the method is mainly due to the easy production of large amounts of antiserum, which need not be purified, and to the need for only small volumes of antiserum in the assay. Laser immunonephelometry costs about $30 per 100 samples, in duplicate, which can be performed in one 8-h working day. Analysis of the same number of samples measured by radioimmunoassay would cost about $500.

Urine is often stored for long periods at −20 °C, particularly in research programs. We found albumin concentrations to be decreased in frozen urine samples, especially in those in which precipitates (mainly urates) had formed. Presumably, albumin is entrapped in the precipitates; however, heating five precipitate-containing samples for 1–12 h at 37 and 56 °C did not restore the albumin concentration to its original value.

It has also been suggested that urinary pH is important in the formation of precipitate leading to decreased albumin concentrations in urine samples (8), but we found no relation between pH and precipitate formation.

We found no loss of urinary albumin when samples were analyzed within two weeks and stored at 4 °C. Because important conclusions are sometimes drawn from rather small variations in albuminuria, one must be aware that storage at −20 °C can influence albumin concentration. In a concurrent study, Erman et al. (9) found a decrease in urinary albumin concentration after even as little as one week of storage at −20 °C. We confirm that albumin concentrations are underestimated in frozen samples. Two of our samples stored at −20 °C for two weeks showed substantial loss of albumin, but greater differences were seen after two and six months of frozen storage.

References

Concentrations of Carotenoids, Retinol, and Tocopherol in Plasma, in Response to Ingestion of a Meal

Ellen D. Brown, Alice Rose, Neal Craft, Karen E. Seidel, and J. C. Smith, Jr.1

Field studies and epidemiological surveys may necessitate obtaining a blood sample from a nonfasted subject for nutritional assessments. We measured the effect of a standardized test meal, eaten after an overnight fast, on the concentrations of seven carotenoid fractions, retinol, and tocopherol in plasma of eight healthy adults. The 750-calorie test meal did not alter the measured concentrations. We conclude that blood sampled up to 4 h after breakfast can be validly used for these measurements.

Recent interest in the role of carotenoids, retinoids, and tocopherol in cancer prevention has stimulated a number of large surveys. One aspect that must be considered in determining concentrations of these analytes in the blood is the effect of food ingestion. Samples collected during the fasted state are ideal, but it is more convenient to obtain blood throughout the morning from subjects, whether or not they have eaten a breakfast. Furthermore, when studies are compared, there are often differences in the times of blood collection and the fasting state of subjects. Thus, the influence of a meal on these analytes becomes a consideration in the design and interpretation of many investigations.

High-performance liquid-chromatographic (HPLC) methods to measure retinol and tocopherol (1) and individual carotenoids (2) more sensitively and specifically have been reported from this laboratory. Here we have used the new methods to determine whether ingestion of a meal after an overnight fast affects the concentration of these nutrients in plasma. We also measured triglyceride and cholesterol concentrations in plasma as common indicators of lipid metabolism.

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