tion indicate that some cyclosporine metabolites—particularly M1, M13, M17, M18, and M21—possess immunosuppressive activity as demonstrated in vitro by use of lymphocyte-proliferation assays. Metabolites M17 and M1 in particular exhibit immunosuppressive activity in vitro approaching that produced by cyclosporine. Moreover, these metabolites appear to exhibit varying immunosuppressive potency depending upon the T-cell clones used in the test system (8, 9). Therefore, the drug concentrations measured by the FPIA assay may more closely approximate the concentration of immunosuppressive drug present in the circulation. These differences between the two assay systems, coupled with the fact that there is greater imprecision with the RIA method, account for the observed correlation coefficient of 0.884 as well as the greater scatter observed at high values.

There are two potential problems with the TDx plasma cyclosporine assay: (a) Assay linearity of 1000 μg/L was not sufficient in our laboratory because about four to six samples in a tray of 20 needed to be diluted. (b) With prolonged use, the integrity of the kit may be compromised because, over time, control values may exceed the high limit, possibly owing to some evaporation of the reagent. However, we observed no substantial evaporation during an analytical run: control values at the beginning and end of several assays were 247 ± 9.6 and 243 ± 9.4 mg/L (n = 24), respectively.

The TDx method offers a simpler procedure with fewer handling steps and faster turnaround time. The instrumentation is automated and compact, allowing better use of technologist time and laboratory space. Reagents and supplies present minimal storage and disposal problems and no radioactivity hazard.

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CLIN. CHEM. 34/9, 1906–1908 (1988)

Glycated Lysine Increases in Serum Samples Stored at −20 °C

Laauge Schäffer

The glycated lysine in serum protein was determined by HPLC measurement of furosine in hydrolyzed serum, with synthetic N₆-Boc-N₁-(1-deoxy-o-fructosyl)-L-lysine as the standard. The glycated lysine value increased with time of storage when the serum was kept at −20 °C, possibly because of an ongoing reaction between glucose and the ε-amino groups of lysine in the frozen serum. The problem was eliminated by using storage at −70 °C or by acidifying the serum before storing it at −20 °C.

Additional Keyphrases: sample handling · variation, source of · glycated proteins · diabetes

Determination of glycated albumin or glycated serum protein as an index of diabetic control over the preceding two to three weeks has been proposed as a useful supplement to the determination of glycated hemoglobin, which reflects the control over the preceding four to six weeks (1–3). Glycated serum protein has been determined by various color reactions (1, 4, 5), by affinity chromatography (6), and by determination of furosine in hydrolyzed serum (7, 8). All the methods require that samples be analyzed shortly after collection. Here I show why this is so, and propose storage conditions under which samples can validly be kept longer.

Materials and Methods

Sample preparation. A mixture of 100 μL of serum and 500 μL of 9.6 mol/L HCl was hydrolyzed at 95 °C for 20 h in a 1.5-mL Eppendorf plastic vial. The hydrolysate was then partly neutralized with 800 μL of saturated sodium formate.

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NH₂–CH–COOH

(CH₃)₄

NH

CH₃

C = O

NH

CHOH

CHOH

CHOH

CH₂OH

Glycated lysine

Furosine

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With every batch of serum samples to be hydrolyzed, I included a standard containing l-phenylalanine and N-boc-N-(1-deoxy-d-fructosyl)-l-lysine (9), treating it exactly like the serum samples. The phenylalanine values were routinely determined, as a rough measure of total protein concentration, to identify samples from patients with gross protein disorders.

**HPLC system.** A Spectrophysics SP 8100 Liquid Chromatograph equipped with two consecutive spectrophotometric detectors, SP 8300 and SP 8440, and an SP 4200 computing integrator was used. The 200 × 3 mm Spherisorb ODS column (Chrompack, Middelburg, The Netherlands) was equipped with a 10-mm guard column. The eluent was 60 mmol/L citrate buffer, pH 4.5, containing 3.0 mmol of sodium l-heptanesulphonate (Sigma Chemical Co., St. Louis, MO) per liter. The column temperature was 40 °C, the flow rate 1.2 mL/min, and the injection volume 10 μL. Absorbance was recorded at 280 nm (furosine, retention time 7.6 min) and at 254 nm (phenylalanine, retention time 3.5 min).

All samples were analyzed in duplicate. The within-run CV was 2.2% and the between-run CV 5.2%.

**Storage of serum.** Serum from a nondiabetic person was stored under different conditions: untreated, −20 °C; with added glucose, 1 mg/mL, −20 °C; with added glucose, 2 mg/mL, −20 °C; with added citric acid, 10 mg/mL, −20 °C; with added glucose, 2 mg/mL, and citric acid, 10 mg/mL, −20 °C; and untreated, −70 °C. Furosine was determined after 19, 34, 58, and 111 days of storage.

In another experiment, serum samples from nine nondiabetic persons were stored at −20 °C, either untreated or with an added crystall of citric acid, lowering the pH to about 5. Furosine was determined after 45 days. Control samples from these subjects were analyzed within 48 h of collection.

**Results**

Figure 1 shows the results for serum with and without added glucose. The increase in glycated lysine at −20 °C correlated strongly with the glucose concentration. If the pH of the serum was lowered to about 5 by adding citric acid, there was no detectable increase in glycated lysine after four months at −20 °C, even with added glucose. When serum was stored at −70 °C, the glycated lysine value remained constant, regardless of pretreatment.

Figure 2 shows results obtained for nine different sera stored at −20 °C, with and without citric acid.

**Discussion**

The furosine method is probably the most reliable for determining glycated protein in serum. Based on a well-defined chemical reaction (10), it overcomes the lack of specificity of some of the more empirical methods. Thus, the results obtained with this method for investigating the effects of sample storage should be valid for any of the other methods. The increase in glycated lysine in serum samples that had been stored at −20 °C could be due either to an ongoing reaction between glucose and e-amino groups of lysine in the frozen sample or to some kind of rearrangement of the glycated lysine already formed, to produce a species giving a higher yield of furosine upon hydrolysis. That the first explanation is more probable was shown by the correlation between the amount of added glucose and the increase in glycated lysine.

The glycation reaction consists of two steps, Schiff base formation and Amadori rearrangement. The equilbrium concentration of the Schiff base increases with increasing pH, whereas the Amadori rearrangement is catalyzed by acid (11). The combination of these two factors gives a pH optimum that is within the physiological range. It is not known whether the glycation reaction in frozen serum involves both reaction steps or only the Amadori rearrangement of the Schiff base, considerable quantities of which might be formed during the freezing of the sample.

The glycation reaction was prevented by storing the serum at −70 °C, but I also found a more generally useful solution to the problem, taking advantage of the pH-dependence of the Schiff base equilbrium. Acidification of the serum with citric acid (5–10 g/L) before storage effectively inhibited the glycation reaction at −20 °C, even in the presence of high concentrations of glucose. Following this rather simple expedient, one can store serum for at least three months without affecting results of furosine assays. This could be useful for some long-term research projects, such as family studies of pre-diabetes, where samples could be collected over some period of time and analyzed at the end of the study.

**References**


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Fluorimetry of Selenium in Body Fluids after Digestion with Nitric Acid, Magnesium Nitrate Hexahydrate, and Hydrochloric Acid

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A digestion procedure involving nitric acid, magnesium nitrate hexahydrate, and hydrochloric acid suffices for selenium determinations in whole blood, serum, and urine by molecular fluorescence spectrometry. To test the accuracy of the method we compared the results with those from hydride-generation atomic absorption spectrometry, and we also analyzed reference materials.

Additional Keyphrases: trace elements · molecular fluorescence spectrometry · hydride-generation atomic absorption spectrometry compared · urine

About half of those laboratories involved in selenium determinations in blood perform their analyses by molecular fluorescence spectrometry (MFS) (1, 2). The procedure is usually based on the reaction between tetravalent selenium and 2,3-diaminonaphthalene at a pH between 1 and 2. A fluorescent complex, 4,5-benzopiazeleno, is formed, which is extracted into an organic phase (usually cyclohexane), where its fluorescence is measured. Most often, the sample digestion is performed with an acid mixture including perchloric acid (e.g., 3). Efforts to replace this hazardous but effective agent for destruction of biological material include trials of a combination of phosphoric acid, nitric acid, and hydrogen peroxide (4). A digestion procedure based on a mixture of magnesium nitrate, nitric acid, and hydrochloric acid for the determination of selenium in blood and urine samples has proved suitable when combined with hydride-generation atomic absorption spectrometry (HG-AAS) (5). Here we report the compatibility of this digestion procedure with the well-established MFS method for determining selenium.

Materials and Methods

Apparatus

We used the HG-AAS instrumentation described previously (6), a fluorescence spectrometer (Perkin-Elmer Model 204) equipped with a xenon lamp, and 1.0-cm (pathlength) quartz cuvettes for all measurements.

A temperature-regulated aluminum block (6) was used for digestion, reduction, and reaction.

Reagents and Materials

All acids and reagents were of “pro analysi” quality except for the diaminonaphthalene (Fluka Chemie AG, Buchs, Switzerland), which had to be purified just before use as follows. To 50 mL of de-aerated 0.1 mol/L HCl add 50 mg of diaminonaphthalene and 250 mg of hydroxylammonium chloride. Heat this mixture at 50 °C for 15 min, cool, and filter the solution into a separatory funnel. Add 10 mL of cyclohexane, shake for 1 min, and let the phases separate for 5 min. Filter the aqueous (lower) phase into a second funnel containing 10 mL of cyclohexane and repeat the extraction. This time, filter the aqueous phase into a 50-mL screw-capped test tube. Bubble nitrogen gas through the mixture for 2 min, and cap the tube. To the indicator solution was prepared by dissolving 0.3 g of gentian violet in 100 mL of water; the 36 mmol/L EDTA solution was prepared by dissolving 6.7 g of Na2EDTA · H2O in 500 mL of water.

Selenium standard solutions were prepared as described in ref. 6.

The reference materials were Standard Reference Material no. 1577a (bovine liver; U.S. National Bureau of Standards, Gaithersburg, MD) and Seronorm 105 (human serum) and 108 (human urine) from Nycomed AS, Oslo, Norway.

The filter paper used was of medium pore size (no. 00M; Munktell, Grycksbo, Sweden).