Glycosylated Hemoglobin Measured by Affinity Chromatography: Micro-Sample Collection and Room-Temperature Storage

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Under proper conditions, whole blood can be stored at room temperature for as long as 21 days before measurement of glycosylated hemoglobin by affinity chromatography. Whole blood (anticoagulated with EDTA or heparin) was placed in capillary tubes, which were then sealed at both ends and stored at room temperature. Just before assay, whole blood was rinsed from the tubes and diluted 10-fold with water. Samples of each patient's blood were assayed as whole-blood hemolysates by affinity chromatography after zero, seven, 14, and 21 days of storage. Values for glycosylated hemoglobin did not change over 21 days of storage and values for each storage day correlated well (r = 0.97, p < .0001) with hemoglobin A1c measured in fresh erythrocyte hemolysates by “high-performance” liquid ion-exchange chromatography.

Additional Keyphrases: screening · diabetes

Glycosylated hemoglobin is now widely measured as an index of long-term glycemic control in diabetic patients (1). We recently reported the use of m-aminophenylboronic acid affinity chromatography to measure glycosylated hemoglobin (2). This method has certain important advantages for the clinical laboratory over previously described techniques in that it is relatively insensitive to changes in temperature and is not affected by labile hemoglobin fractions (2). In another report (Little et al., pp 1112–1114, this issue) we showed excellent stability of affinity chromatography values for whole blood stored for up to eight days at room temperature, but with increases in values thereafter. In that study, erythrocytes from stored whole blood were washed and incubated before assay. In this report, we show that whole blood hemolysates prepared before assay, without washing or incubation of erythrocytes, show stability for at least 21 days. We also give data on a micro-scale method for blood sample collection that is well suited to this affinity method.

Materials and Methods

We obtained blood specimens from 34 diabetic and non-diabetic subjects by venipuncture and collection in EDTA-containing tubes. Part of each specimen was transferred to four Caraway capillary tubes (300–350 µL capacity), which were then sealed with putty sealant (CritoSeal; American Scientific, St. Louis, MO 63043) on one end and ParaFilm on both ends. Three of the capillary tubes were then stored at room temperature (20–24 °C) until assayed seven, 14, and 21 days later. The fourth capillary tube from each subject was assayed by affinity chromatography on the same day the blood was collected. Nine of the 34 specimens collected in EDTA were also collected in heparinized tubes.

Five additional specimens of capillary blood were collected via finger-stick directly into standard heparinized microhematocrit capillary tubes. These capillary tubes were then sealed at both ends with plastic caps (CritoCaps, American Scientific).

To determine the effects of glucose on affinity chromatography results before and after storage of whole blood, some samples were supplemented with glucose to give final plasma glucose concentrations ranging from 0.93 to 13.6 g/L. An aliquot of each sample was then assayed and the remaining samples were stored at room temperature in capillary tubes until assay seven and 14 days after collection.

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Received Dec. 27, 1982; accepted Mar. 7, 1983.
Just before assay, whole blood was washed from the capillary tubes with water and finally diluted about 10-fold. Glycosylated hemoglobin was measured with the Glycosylated Hemoglobin Clinovative Diagnostic Kit (Pierce Chemical Co., Rockford, IL 61105). Fifty microliters of the diluted hemolsate was applied directly onto each column, and each sample was assayed in duplicate according to instructions provided in the kit. For quality control, pooled hemolsates stored at -70 °C from diabetic and non-diabetic subjects were assayed with each batch of samples.

For comparison with affinity chromatography results, we used an aliquot of each fresh whole-blood sample to prepare an erythrocyte hemolsate for analysis for hemoglobin A1c by a semi-automated "high-performance" liquid-chromatographic ion-exchange method (3). These hemolsates were stored at -70 °C until analysis.

Results

Values for glycosylated hemoglobin in diabetic and non-diabetic samples stored as whole blood in capillary tubes showed no appreciable change in glycosylated hemoglobin after 21 days at room temperature. The mean glycosylated hemoglobin values (± SEM) of 34 whole-blood samples were 13.45 ± 0.59, 13.62 ± 0.60, 13.30 ± 0.58, and 13.22 ± 0.61 on days zero, seven, 14, and 21, respectively. None of the differences is significant. Figure 1 shows the relationship between individual glycosylated hemoglobin values obtained on day 21 vs day 0. The regression line (y = 1.03x - 0.50) falls very close to the unity line and individual variability around these lines is relatively small. The average coefficient of variation between stored whole-blood samples assayed on different days was 3%. The inter-assay CVs for the pooled diabetic and non-diabetic hemolsates were 3.05% and 2.59%, respectively. There were also no significant differences in glycosylated hemoglobin values between paired EDTA-treated and heparinized blood samples stored for 21 days.

Glycosylated hemoglobin as measured by affinity chromatography on each of the storage days correlated strongly with hemoglobin A1c measured in fresh hemolsates by ion-exchange chromatography, an established method (y = 1.53x - 0.996, all r values = 0.97, p < .0001).

Results by the affinity chromatography method do not appear to be affected directly by variations in glucose concentration in the range we studied. However, after storage at room temperature for 14 days, blood samples with plasma glucose values greater than 6.72 g/L showed significant increases in glycosylated hemoglobin (Table 1).

Discussion

Many techniques are available for measuring glycosylated hemoglobin. For the clinical laboratory, the choice of a method can be very difficult. Important considerations in selecting an appropriate method include its accuracy, equipment costs, extent of interference by labile fractions, and temperature and pH sensitivity. Other important considerations include convenience of sample collection, sample storage stability, and assay speed (4).

We and others have shown that sample storage conditions and sample preparation before assay are critical for most assay techniques. For ion-exchange and electrophoretic methods, it is necessary to remove the labile fraction (pre-A1c) which co-chromatographs or co-migrates with the stable glycosylated hemoglobin (3, 5). Ion-exchange and electrophoretic results are also affected by artifactual increases in the hemoglobin A1c fraction during storage of whole blood or hemolsates at room temperature (3, 6). Analysis for glycosylated hemoglobin by the thiobarbituric acid colorimetric method requires that glucose be completely removed before assay (4).

Affinity chromatography is technically simple and is not significantly affected by the above factors. Our results show that it is feasible to measure glycosylated hemoglobin by affinity chromatography in whole blood collected in capillary tubes and stored at room temperature for long intervals without removing glucose or labile glycosylated hemoglobin before assay. As little as 10 μL of whole blood collected by finger stick suffices for assay in duplicate, and either EDTA or heparin can be used as an anticoagulant. The method is easily adaptable to large-volume batch assays, the average assay time being about 6 min per duplicate sample. Results are not affected appreciably by sample glucose concentration unless it exceeds 6.7 g/L. One important technical consideration is that samples not be allowed to dry during storage to avoid in vitro glycosylation with spuriously increased test results (7). Furthermore, for storage at room temperature, stability is greatest when hemolsates are prepared directly from whole blood, without washing the erythrocytes or incubation with saline before assay.

With this assay method, the ease of sample collection and the storage stability of samples have important clinical applications aside from the direct advantages for the clinical
laboratory. This method may be particularly well suited to large diabetic screening studies and as an adjunct to routine diabetes care where patients are carrying out home monitoring of capillary blood glucose. In this situation, patients could collect whole-blood samples at home and send them directly to the central laboratory.

This study was supported in part by USPHS Research Grant HL-13832. Our thanks to Mrs. Betty Payne for her technical assistance in the preparation of this manuscript.

References


CLIN. CHEM. 29/6, 1082–1084 (1983)

Liquid-Chromatographic Study of Fluorescent Materials in Uremic Fluids
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Using reversed-phase "high-performance" liquid chromatography with fluorescence detection, we separated and identified some naturally fluorescent compounds in uremic serum and hemodialysate from patients with chronic renal disease. Several of the naturally fluorescent compounds were identified as indole derivatives by co-chromatography with authentic standards. In one specific case, the identity was confirmed by an enzymic peak-shift method. Compounds identified included indican, kynurenic acid, tryptophan, and 5-hydroxyindole-3-acetic acid. Comparison of normal and uremic serum showed that the fluorescent materials are present in significantly greater concentrations in samples from uremic patients.

Additional Keyphrases: chromatography, reversed-phase · hemodialysis patients · chronic renal disease · indole derivatives · enzymic peak-shift method

Recent Letters to this journal have discussed the presence of unidentified naturally fluorescent compounds in the serum, urine, hemodialysate, and hemofiltrate of patients with chronic renal failure (1–4). Vladutiu et al. (1) reported finding one or more fluorescent substances (maximum emission near 340 nm) in the serum of patients with chronic renal failure but not in the serum of those with acute renal failure. They concluded that the substances were not vitamins or anabolic steroids and could be bound to a serum protein. Schwertner et al. (2) found a fluorescent compound (excitation maximum 322 nm, emission maximum 415 nm) not only in serum, but also in hemofiltrate, hemodialysate, and urine of patients with chronic renal disease. The substance was 80-fold more concentrated in uremic serum than in normal serum. They also determined (by gel filtration) that the relative molecular mass (M₉) of the substance was probably <1000, and that it was not a drug or drug metabolite. Digenis et al. (4), finding a linear correlation between the relative fluorescent intensity and the creatinine concentration in serum from patients with chronic renal failure, concluded that deterioration of renal function is accompanied by an increase in the unidentified fluorescent substances.

We have separated and identified some naturally fluorescent compounds in uremic serum and hemodialysate, by use of reversed-phase "high-performance" liquid chromatography (HPLC) with fluorescence detection. We also used a unique enzymic peak-shift identification procedure.

Materials and Methods

Materials

Apparatus. The mobile phase was pumped through the apparatus with an SP8700 solvent-delivery system (Spectra-Physics, Santa Clara, CA 95051). Samples were injected via a syringe loading valve fitted with a 100-μL sample loop (Model 7120; Rheodyne Inc., Berkeley, CA 94710) onto a 3.9 × 300 mm stainless-steel column containing 7 μm Zorbax ODS (Du Pont Co., Wilmington, DE 19898) packing material. The compounds of interest were detected with an Aminco filter fluorometer (American Instrument Co., Silver Spring, MD 20910) equipped with a G.E. germicidal lamp (Model J4-7126), a Warrten 2C emission filter (peak wavelength 405 nm, approximate bandpass 10 nm), and an Amino J4-7469 excitation filter (peak wavelength 295.4 nm, bandwidth at 50% of maximum transmission 9.2 nm). All chromatograms, retention times, and peak areas were recorded with an SP4100 computing integrator (Spectra-Physics).

Water was purified by passage through a Milli-R04 system, followed by a final cleanup through a Milli-Q system (Millipore Corp., Bedford, MA 01730). Proteins (M₉,