organic extraction is used to separate retinol from RBP. The binding of retinol to RBP, which binds retinol specifically, enhances retinol fluorescence by about 10-fold.

The Futterman method must be standardized with serum of known retinol concentration. Other retinol methods, all of which involve extraction with organic solvent, may be standardized with pure retinol. This necessity is an advantage in one way: endogenous serum retinol is completely stable for years when the serum is stored at about 0°C (9), whereas pure retinol begins to degrade in months.

Although not useful for large national surveys and clinical trials because of the possibility for interferences, the Futterman method has the advantages of simplicity and economy with good precision and reasonable accuracy. It is thus well suited for situations where laboratory technology and facilities are limited.

We are indebted to Mr. Wayman Turner for advice on statistical analysis. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Dept. of Health and Human Services.

References

Collection of Blood on Filter Paper for Measurement of Glycated Hemoglobin by Affinity Chromatography
Randle R. Little,¹ Edith M. McKenzie,¹ Hsiao-Mei Wiedmeyer,² Jack D. England,² and David E. Goldstein¹,²

We present data on a filter-paper collection and assay method for measurement of glycated hemoglobin (gHb). Onto filter paper dipped into a solution of glucose oxidase (EC 1.1.3.4) and allowed to dry, approximately 20 µL of capillary blood was spotted. For analysis, we eluted the dried blood spot from the paper by soaking in water for 1 h, then measured the gHb in the eluate by affinity chromatography. Because the gHb significantly increased from day 1 to day 14 of storage, it was necessary to standardize the day of elution from the paper. We found a high correlation between gHb measured from samples stored for 14 days on treated paper and gHb measured by affinity chromatography from frozen whole blood or hemolysates (r = 0.96). This method is convenient, requiring small amounts of blood and little sample handling and assay time, and may be particularly useful in certain situations such as large-scale screening for diabetes.

Additional Keyphrases: variation, source of - screening - diabetes

We present data on a filter-paper collection and assay method for measurement of glycated hemoglobin (gHb) in whole blood (glycated hemoglobin and glycated plasma proteins) (6). In that method, about 120 µL of blood was spotted directly onto filter paper, which was then washed with alcohol before being mailed to the laboratory. Blood eluted from the paper was analyzed for glycated whole blood proteins by a thiobarbituric acid colorimetric method. Here we present data on a different filter-paper collection and assay method that requires less blood and less time for sample handling and assay.

Materials and Methods
Blood samples. Subjects for the study were type I and type II diabetic patients and non-diabetic volunteers from the University of Missouri Health Sciences Center in Columbia.

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Filter papers (no. 903; Schleicher and Schuell, Keene, NH 03431) were soaked in a glucose oxidase (EC 1.1.3.4)-containing solution (Beckman Glucose Reagent; Beckman Instruments, Fullerton, CA 92634) for about 2 min and allowed to air-dry (7). Venous blood samples were collected in evacuated collection tubes containing EDTA, then spotted onto the treated filter paper. Capillary blood samples were obtained by fingerpunt with a spring-loaded sterile lancet (Autolet and lancets; Owen Mumford Ltds., Woodstock, Oxford, U.K.) and collected by gently applying a few drops of blood to the treated filter paper. The papers were then allowed to dry at room temperature. An aliquot of each whole-blood sample was stored at −70°C; another aliquot of whole blood was reserved for preparation of erythrocyte hemolysate. To determine the effects of storage temperature on gHb results, we kept the blood-spotted filter paper at 4, 22, 30, and 37°C for one to 21 days.

Preparation of samples. To prepare the erythrocyte hemolysates for analysis of Hb A1c by “high-performance” liquid chromatography (HPLC), centrifuge the venous blood (1500 × g, 10 min, 4°C), then remove the plasma and the leukocyte layer. Wash the packed cells three times, each with five volumes of isotonic saline, then incubate in 12 volumes of saline 37°C for 5 h. Centrifuge, remove the saline, and lyse the packed cells with two volumes of distilled water. Add one volume of carbon tetrachloride to the hemolysate, vortex-mix, and refrigerate at 4°C overnight. After centrifuging the hemolysate (27 000 × g, 30 min, 4°C), store the aqueous supernate at −70°C until analysis.

To prepare the samples for analysis for gHb from dried blood samples, remove the dried blood spots with a standard 6-mm-diameter paper punch. Elute the blood from the filter paper disks for 1 h with water (150 µL of water for each 6-mm disk or for each 20 µL of whole blood). Remove the paper and store the eluate at −70°C until assay.

Assay procedures. Our HPLC apparatus and procedure used in the measurement of Hb A1c have been described previously (6).

For affinity chromatography we used a commercially available kit (“GlycoTest”; Fierce Chemical Co., Rockford, IL 61105) according to the manufacturer’s instructions. The samples were filter-paper eluates, applied directly to the column without dilution; corresponding whole-blood samples that had been diluted 10-fold with water; and hemolysates (20-fold, final dilution of erythrocytes with water). For assays in duplicate, at least 20 µL of whole blood had to be spotted on the paper.

For quality control of our affinity chromatography assay, we assayed in duplicate with each batch of samples, two pooled hemolysates (stored at −70°C), one from diabetic and one from nondiabetic subjects.

Results

We have previously shown that drying and storage of whole blood on filter paper increased the final measured value of glycated whole blood proteins by thiobarbituric acid colorimetry, reflecting the rapid in vitro glycation of hemoglobin and plasma proteins during drying (6). Our present results show a similar increase in gHb measured by affinity chromatography after storage of blood on untreated paper. Pretreatment of the filter paper with a glucose oxidase-containing reagent substantially lowered but did not completely inhibit the increase in gHb over time. Glycated Hb increased significantly from day 1 to day 14 but did not increase further up to day 21 (n = 28; % gHb = 11.57, 12.61, 13.28, and 13.46 for days 1, 7, 14, and 21, respectively; p = 0.07, day 14 vs 21, paired t-test).

We studied the mechanism of the increase in gHb over time as measured by affinity chromatography from samples dried on treated paper. The percentage increase between day 1 and day 7 showed a very low negative correlation with blood-sample glucose concentration (r = −0.38, p < 0.02) or with the gHb values from a fresh (nondried) sample (r = −0.38, p < 0.01). Pretreating an hemolysate to remove glucose and labile gHb (9) before spotting it onto the treated paper resulted in a much smaller average increase in gHb from day 1 to day 14 than when whole blood was spotted directly on paper (4.56 vs 14.9% increase). Adding glucose to an hemolysate before applying it to the paper produced a greater increase in gHb from day 1 to day 14 for high glucose concentrations (60 mg/L). A very large labile component (2.7% pre-A1c, created by incubating erythrocytes with high concentrations of glucose before sample application) (9) also increased the value at day 14.

Because a substantial increase in the gHb value occurred between day 1 and day 14 of storage on treated filter paper but not thereafter, we standardized the length of storage (i.e., number of days between sample application to and sample elution from the paper) for this collection and assay method. We chose to elute on day 14 because, by that time, the value should have plateaued and samples being transported should have reached the laboratory.

We assessed the reproducibility of this filter-paper collection and storage method by assaying an eluate from a frozen whole-blood control sample that had been spotted onto filter paper on 12 separate occasions. The CV was 5.28% (mean gHb = 7.21%, SD = 0.36%). Reproducibility was also evaluated by assaying two separate samples from each of 15 diabetic patients; one sample was collected by a nurse in the clinic, the second was collected within a few days by the patient at home and mailed to the laboratory. The CVs for these “duplicate” samples assayed together ranged from 0.16 to 5.63%, with no significant difference (p > 0.1, paired t-test) between the two groups of samples.

The normal range (mean ± 2SD) for % gHb, measured from 26 nondiabetic volunteers by using this method with day 14 elution, was 8.66 ± 1.60%. The normal reference interval (n = 28, mean ± 2 SD) for % gHb, as measured by affinity chromatography of hemolysates (no drying on paper), was 5.83 ± 1.48%.

Figure 1 shows the high correlation (r = 0.96, p < 0.0001) between gHb measured from stored filter-paper samples and gHb from frozen whole blood or erythrocyte hemolysates, both measured by affinity chromatography. Quadrants I through IV indicate the upper limits of normal (mean ± 2SD) for each method. In only one case there was a significant discrepancy between results for stored filter-paper samples and stored frozen samples (Quadrant IV). The correlation between gHb measured by affinity chromatography from stored filter-paper samples and Hb A1c measured by HPLC from frozen hemolysates was also high: r = 0.89 (p < 0.0001).

Figure 2 illustrates the effect of 21 days of storage at various temperatures on results for samples from three individuals. Only storage at 4°C had any consistent effect on gHb values between samples after 14 days. When we examined the effect of storage at 4, 22, and 37°C for three or four days (an average transport time for mailed samples),
method, in which we use a glucose oxidase-containing reagent to bind free glucose, has several advantages over the previous method. Pretreatment of the filter paper eliminates the need for any sample treatment after collection but before transport. The assay by affinity chromatography requires collection of only very small sample volumes (20 μL of whole blood). In addition, the assay itself is technically easier and faster than the previously described method.

The observed increase in gHb from glucose oxidase-treated paper is not easily explained. The gHb in erythrocyte hemolysate samples stored on treated paper is increased even when glucose and labile gHb components are removed before the sample is spotted onto the paper. Furthermore, in our samples, the increase in gHb with storage was not positively correlated with the glucose concentration. The increase can, however, be exaggerated by supplementing samples with high amounts of either glucose or labile gHb. Therefore, the increase in measured gHb may be partially due to in vitro glycation from residual glucose in the sample, or from conversion of labile Schiff base components to stable gHb, but we cannot show a clear relationship between these factors and our final measured values in the corresponding whole-blood samples.

The reproducibility of this filter-paper collection and storage technique is excellent (CV = 5.28%) if the day of elution from the paper is standardized. This filter-paper method is convenient for patients and technicians, and results correlate well with those of a well-established gHb method. This test may be particularly useful in clinical situations such as patient care in remote areas and screening for diabetes.

References


Discussion

The convenience of blood-sample collection and handling and the effects of sample storage are important factors in the use of gHb for both routine patient care and large-scale screening programs. Our previously reported filter-paper method (5) required 120 μL of whole blood and prevented in vitro glycation by treatment with alcohol to remove glucose from blood spots. Despite its limitations, the method was useful for a large-scale screening study (10). The present

Fig. 1. Correlation between % gHb measured by affinity chromatography from frozen blood samples and % gHb measured by affinity chromatography after storage on treated filter paper for 14 days. The boundaries of Quadrants I–IV are the upper limits of the normal range (mean ± 2SD) for each method.

Fig. 2. Effect of temperature on % gHb in three diabetic individuals. Dried samples were stored at 4 °C (– – – –), 22 °C (O—O), 30 °C (—– —), and 37 °C (O—O), until the day of elution with 22 °C storage thereafter, we found that the time-dependent increase in gHb was not related to temperature.