Measurement of Glycosylated Whole-Blood Protein for Assessing Glucose Control in Diabetes: Collection and Storage of Capillary Blood on Filter Paper

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We present data on the use of filter-paper blood collection for measurement of glycosylated whole-blood proteins (gWB) (hemoglobin and plasma proteins). A capillary blood sample, obtained by fingerprick, is spotted directly onto filter paper (Schleicher & Schuell 903). The blood spot is washed briefly with alcohol (ethanol or isopropanol) to remove free glucose and dried before shipment to the laboratory. In the laboratory, the blood is eluted from the paper and analyzed for gWB by a colorimetric method. The gWB is primarily a measure of glycosylated hemoglobin (gHb) with a small contribution from glycosylated plasma protein. Concentrations of gWB and gHb are highly correlated (r = 0.91). The filter-paper method offers advantages over currently available methods for quantifying gHb and may be particularly useful in screening for diabetes and for assessing glycemic control in patients from remote areas.

Additional Keyphrases: glycohemoglobin • glycoproteins • sample handling • screening

Glycosylated (glycated) hemoglobin and plasma proteins are increased in patients with diabetes mellitus (1). The actual concentration of a particular glycosylated protein reflects both the time-integrated glucose concentration and the turnover time of the protein. Determination of glycosylated hemoglobin is considered an accurate index of the mean blood glucose during the preceding two to three months; glycosylated plasma protein reflects blood glucose concentration during a shorter period, two to four weeks, given the shorter turnover time of plasma proteins (2).

Measurement of glycosylated hemoglobin (gHb) and glycosylated plasma proteins (gPP) is limited by the inconvenience of sample collection, which usually requires venipuncture.4 In addition, many sample handling and storage factors can markedly alter assay results (3–5). These factors can be a major concern in certain clinical settings, such as large-scale screening programs and patient care in remote areas. In this report we present data on the use of filter-paper collection of blood by fingerprick and determination of glycosylated whole-blood protein (gWB), a measure that reflects glycosylation of both hemoglobin and plasma proteins.

Materials and Methods

Blood Sample Collection

Subjects for this study were type I and type II diabetic patients and nondiabetic volunteers from the University of Missouri Health Sciences Center in Columbia, Missouri, and from central Arizona. Most of the Arizona subjects were Pima Indians who were participants in long-term longitudinal studies conducted by the Southwestern Field Studies Section of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. Venous and capillary blood samples were obtained from all subjects.

Venous blood samples were collected in EDTA-containing evacuated blood-collection tubes. Blood specimens collected in Arizona were shipped to Missouri on ice via an overnight delivery service. In some of the subjects blood samples were collected serially over a four-month period.

Capillary blood samples were obtained by fingerprick with a spring-loaded sterile lancet (Autolet and lancets; Owen Mumford Ltd., Woodstock, Oxford, U.K.) and collected by gently applying the drops of blood to filter paper (no. 903; Schleicher and Schuell, Keene, NH 03431) to fill four 14-mm-diameter circles (about 120 µL of blood). The filter paper containing the blood spots was placed in approximately 100 mL of 700 m/L ethanol or isopropanol for 1 h and occasionally gently agitated. The paper was then allowed to dry at room temperature (19–24 °C). Filter paper samples collected in Arizona were mailed first class to the University of Missouri Health Sciences Center for further analysis.

In some experiments blood was collected in heparinized Caraway capillary tubes, which were then sealed with putty sealant on one end and with Paraflm on both ends and stored at room temperature for up to two weeks.

A whole-blood control sample, stored in small aliquots at −70 °C, was also spotted on filter paper with several different groups of blood samples from patients.

Preparation of Samples

Erythrocyte hemolysate for analysis of hemoglobin A1c by "high-performance" liquid chromatography (HPLC). Centrifuge the venous blood at 3000 × g for 10 min at 4 °C and remove the plasma and the leukocyte layer. Wash the packed cells three times with five volumes of isotonic saline. Add 12 volumes of saline and incubate at 37 °C for 5 h, then centrifuge and remove the saline. 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. Centrifuge the hemolysate at 27 000 × g for 30 min at 4 °C and store the aqueous supernate at −70 °C until analysis.

Samples for analysis of gWB from stored capillary blood samples. For whole-blood spotted on filter paper, elute the blood from the filter paper for 1 h with 2 mL of NaOH, 15 mmol/L. Remove the paper and store the eluates at −70 °C until assay.
Treat whole blood stored in capillary tubes as follows: Rinse whole blood from the tube and lyse erythrocytes with two volumes of distilled water. Remove glucose by precipitating the protein with 12 mL of acidified acetone (6); centrifuge (1700 × g, 10 min, 4 °C), and discard the supernate. Wash the pellet once with 12 mL of acidified acetone, twice with acetone, and once with 7 mL of diethyl ether. Dry the pellet under a stream of nitrogen, dissolve the pellet in 2 mL of water by warming at 37 °C or by sonication and store the protein solution at −20 °C until assay.

**Venous blood for measurement of gWB, gHb, and gPP.** From nine subjects sampled serially over four months, we prepared venous blood samples for measuring gWB, gPP, and gHb by placing 1–2 mL of whole blood, plasma, and erythrocytes (washed once with saline) into separate dialysis bags (3500 M cutoff) and dialyzing in saline for 24 h at 4 °C to remove glucose. We stored the dialyzed samples at −70 °C until assay by colorimetry.

**Assay Procedures**

Our HPLC apparatus and procedure used in the measurement of HbA1c have been described previously (7).

Before determining glycosylated proteins by colorimetry, we measured the total protein by the method of Lowry et al. (8). The colorimetric assay (9) is performed with 10 mg of protein. Each assay includes five fructose standards, a pooled normal hemolysate sample, a pooled diabetic hemolysate sample, and unknowns from patient and control specimens. For each sample, we prepared an appropriate blank, substituting water for thiobarbituric acid, and subtracted the absorbance from that of the corresponding sample. The measured absorbances for the protein samples are converted to fructose equivalents by using a fructose standard curve, with fructose concentrations of 20–100 μmol/L (9).

**Results**

**Relationship between gWB and hemoglobin A1c.** Glycosylated whole-blood protein contains both gHb and gPP. We would expect the contribution of gHb to predominate in the measurement of gWB because of the larger amount (10) and longer half-life of hemoglobin relative to plasma proteins. The relative amounts by weight of hemoglobin and plasma protein in whole blood (10) and the relative contribution from each to total glycosylation in whole blood was determined by measuring gHb, gPP, and gWB in fractionated blood samples from both diabetic and nondiabetic individuals. Approximately 80% of the measured amount of gWB reflects glycosylation of hemoglobin.

Accordingly, there is an excellent correlation (r = 0.91, p < 0.0001) between HbA1c (hemoglobin glycosylated at the N-terminus of the beta chain) measured by an established HPLC method (7) and gWB measured from acid acetone precipitates of fresh whole blood.

**Storage of whole blood on filter paper.** In earlier studies (11) we showed that the drying and storage of whole blood on filter paper increased the final measured value of gWB, and that this increase was directly proportional to the duration of storage and to the concentration of free glucose in the blood spot. The data were most consistent with additional glycosylation of blood proteins during drying and storage (11). In the present study we prevented in vitro glycosylation by removing glucose from the filter-paper blood spot before storage (12); immersion of the blood spot in alcohol allows the glucose to be removed without eluting the blood from the filter paper. Treatment with ethanol or with isopropanol gave comparable results (data not shown). Figure 1 shows the effects of alcohol treatment of the filter-paper blood spots from a single representative blood sample, illustrating the dramatic increase in the gWB value with storage time on filter paper that had not been rinsed with alcohol. Our data suggest that this increase represents in vitro glycosylation of hemoglobin and plasma proteins (11). Figure 1 also shows the stability of whole-blood spots on filter paper that was stored after pretreatment with alcohol. The difference between the alcohol-treated and the untreated samples at time zero reflects a direct effect of the alcohol treatment on the blood spot; the gHb values of all alcohol-treated samples are consistently about 10 fructose equivalents greater than in the untreated samples.

Figure 2 shows the excellent stability of dried alcohol-treated filter-paper samples (n = 61) after one week of storage at room temperature. The mean difference between day 1 and day 7 was 2.56 (SD 3.61) fructose equivalents/10 mg of protein.

**Reproducibility of the filter-paper method.** We assessed the reproducibility of the filter paper/colorimetric assay by...
repeated analysis of an eluate from a whole-blood control sample that was spotted on filter paper and alcohol-treated on 28 separate occasions and measured in 20 separate assays. The CV of 7.2% (x = 62.60, SD = 4.51) is close to the interassay CVs from the pooled normal and diabetic hemolyte controls included in each assay (CVs of 2 to 5%).

Storage in capillary tubes. An alternative to filter-paper storage of blood samples is storage in sealed capillary tubes (13). We found no significant change in gWB measurements after two weeks of storage of whole blood in capillary tubes (data not shown). These samples also show a high correlation (r = 0.92) between gWB and HbA\textsubscript{1c} as measured by HPLC.

Time-course of gWB change: Figure 3 compares gWB, gHb, and gPP in serial determinations from a representative newly diagnosed diabetic patient rapidly brought under metabolic control. The rates of decrease in gWB and gHb were almost identical, whereas gPP declined more rapidly, as expected, reflecting the shorter turnover time of plasma proteins.

Preliminary field trial with the filter-paper method. To determine if the filter paper method would be clinically practical, we carried out a field trial with samples collected from 107 Pima Indians in Arizona, a population exhibiting a very high prevalence of type II diabetes (14, 15). For each subject a venous and a capillary blood sample were collected at the same time and shipped to Missouri as detailed above. Figure 4 shows the excellent correlation (r = 0.92) between measurement of gWB from samples collected on filter paper and HbA\textsubscript{1c}. The normal range (mean ± 2 SD) for gWB in this population by the filter-paper method is 58–79 fructose equivalents/10 mg protein (normal, impaired glucose tolerance, and diabetic groups as defined by WHO criteria based on oral glucose tolerance testing). Long-term studies are underway to further examine the role of gHb and gWB measurement for the diagnosis and screening of diabetes.

Discussion

Collection of capillary blood directly onto filter paper has been used widely and successfully in several clinical situations, most often in newborn screening for metabolic disorders such as phenylketonuria and hypothyroidism. The use of filter paper simplifies blood collection and shipment to the laboratory, and capillary blood specimens are relatively easy to obtain.

Here we report the use of filter-paper blood collection for measurement of glycosylated hemoglobin and plasma proteins incorporated into a single measurement called "glycosylated whole blood protein.” The test, which measures the percent of all blood proteins that are glycosylated, primarily reflects glycosylation of hemoglobin with a small contribution (about 20%) from nonheme proteins. Results correlate highly with HbA\textsubscript{1c} determinations and parallel in vivo changes in HbA\textsubscript{1c}.

The assay itself is a variant of the hydroxymethylfurfural/thiobarbituric acid method first described by Keeney and Bassette (16), modified by Flückiger and Winterhalter (17) and most recently by Parker et al. (9). Compared with other assay methods, this assay has some specific advantages for determination of gHb and gPP, in particular the lack of interference by pre-HbA\textsubscript{1c}, hemoglobinopathies, and nonglucose hemoglobin adducts (9). In addition, fructose can be used as a standard.

Because the colorimetric method is affected by free glucose, and because glycosylation of hemoglobin and plasma proteins proceeds relatively rapidly in vitro during the drying process, we wash the filter paper spot in alcohol shortly after the blood sample is spotted. This removes the free glucose, thereby preventing both in vitro glycosylation and glucose interference in the assay itself. The type of alcohol and the length of alcohol treatment are not critical. At 700 mL/L, either ethanol or isopropanol gives comparable results. Leaving the samples for at least 1 h in alcohol is sufficient to remove even high amounts of glucose.

Use of ion-exchange chromatographic and electrophoretic methods with filter-paper collection of blood has been unsuitable because of marked alteration in the hemoglobin components by drying and storage. Our studies with a boronic acid affinity chromatographic method have not yet yielded consistent results for blood dried on filter paper; a precipitate forms in the eluate during the adjustment of pH that is necessary for the affinity assay.

Eross et al. (18) recently reported that pretreatment of filter paper with a glucose oxidase reagent (Beckman glucose reagent) also prevents in vitro glycosylation. In the past we tried premixing whole blood with glucose oxidase before placing the sample on the filter paper, but found that this technique only partly inhibited the in vitro glycosylation.

Measurement of gWB from filter-paper spots has several useful clinical applications. Because many diabetic patients now include capillary blood monitoring at home as part of their routine diabetes care, specimens could be obtained at home for gWB determinations and then mailed directly to the laboratory. We have already used this approach in various circumstances, e.g., when glycemic control in dia-

![Fig. 3. Rate of change of gWB, compared with that of gHb and gPP, in a newly diagnosed type I diabetic patient, at the beginning of insulin treatment](image)

![Fig. 4. Correlation between gWB measured from filter-paper blood spots and HbA\textsubscript{1c} measured by HPLC: r = 0.13 (not significant) for the nondiabetic subjects, r = 0.92 (p < 0.0001) for the combined diabetic and impaired glucose-intolerance groups](image)
abetic patients must be reassessed before their next regularly scheduled clinic visit, or when they live far from our medical center. Our results suggest that the test may also be useful for diabetes screening.

In summary, our study of the use of filter paper collection of capillary blood for measurement of whole blood glycosylated protein, an index of long-term glycemic control, shows that the blood collection and assay procedures could be well adapted to both diabetes screening programs and routine diabetes care.

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