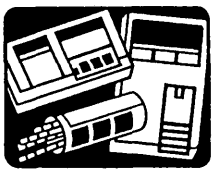
Technical Section



Assessment of Glycosylated Hemoglobin Measurement With Sample Collection Papers

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It would be advantageous to be able to measure glycosylated hemoglobin (GHb) from a sample of blood dried on paper and sent to the physician by the patient. Indeed, such a technique has been introduced by Isolab (Akron, OH). We tested the validity of the method to determine whether *1*) applying the blood to paper and immediately assaying it correlates with the same sample assayed by the usual whole-blood technique, *2*) the size of sample applied to paper affects apparent GHb results, *3*) time and temperature of storage of the sample paper affect apparent GHb results, and *4*) plasma glucose concentration of the sample affects GHb results.

The GHb of samples assayed immediately after application to sample paper versus those assayed as whole blood showed very good correlation ($r = .93$, $P < .001$). Volume of the drop of blood applied to paper (25–100 μl) did not affect results. However, there was a dramatic, temperature-dependent increase in apparent GHb when samples were stored on sample paper, averaging 12.5, 16.3, and 19.5% when stored at room temperature for 3, 6, and 9 days, respectively. Overall, apparent GHb rose from 1.3-fold in 3 days at 4°C to 3.8-fold in 9 days at 37°C. The rate of GHb formation was proportional to plasma glucose concentration, but removal of free glucose by ethanol or glucose oxidase did not yield consistent results for this method. We conclude that these sample papers are not useful as an approach to collecting blood samples for GHb measurement. *Diabetes Care* 10:352–56, 1987

Glycosylated hemoglobin (GHb) provides a useful assessment of long-term blood glucose concentration in diabetes (1–3). Recently, sample papers were introduced that purport to allow measurement of GHb on blood collected from a fingerstick and placed on the absorbent paper (Isolab, Akron, OH). This could offer significant advantages over the current requirement for a venipuncture blood sample. We therefore evaluated the validity of this new approach to GHb assay.

MATERIALS AND METHODS

GHb assays. All GHb measurements were performed with the affinity-chromatography method (Glyc-Affin GHb, Isolab). Samples were analyzed within 24 h of whole-blood collection in EDTA-containing tubes and stored at 4°C until assay. For standard assays from whole blood, 50 μl of blood were hemolyzed in 400 μl of the preparation reagent. For sample-paper assays, papers were used as shipped by Isolab. The characteristics of the paper and its pretreatment were not made available. Samples were eluted from the filter paper by soaking the entire spot of blood on paper in 400 μl preparation reagent for 60 min. Lysates were applied to a microcolumn containing the affinity gel (*m*-aminophenyl boronic acid immobilized on agarose). The nonglycosylated hemoglobins and the glycosylated hemoglobins were eluted successively. Absorbance of each fraction was read in a Gilford spectrophotometer 250 (Oberlin, OH) at 415 nm, and the percentage of total hemoglobin as GHb was calculated. Specificity for hemoglobin and its glycosylated forms is due to measurement at 415 nm (4). Normal range for nondiabetic people is reported by the manufacturer to be 4–8%. All assays were done in duplicate, the results being averaged. Intra-assay coefficient of variation was 2.8%, and interassay coefficient of variation was 4%.

Study samples. Blood samples were obtained from 5 healthy adults with no history of diabetes mellitus and from 45 people with diabetes at the time of routine visits to the Johns Hopkins Diabetes Clinic (samples drawn for routine laboratory assays were split, and half were used for this study). Blood

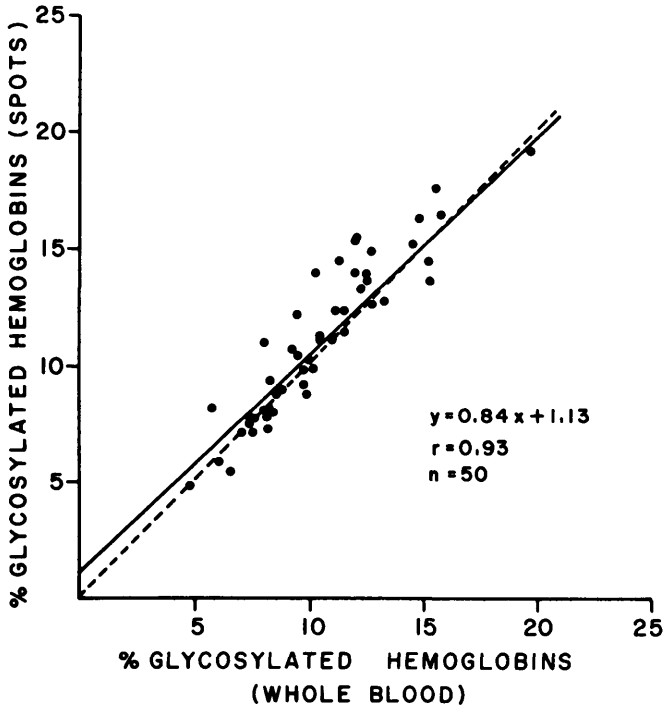

FIG. 1. Correlation between glycosylated hemoglobin (GHb) values determined promptly after venipuncture by affinity-chromatography method on whole blood and on sample papers. *Solid line*, actual least-squares regression line; *broken line*, theoretical line of identity.

was collected in the morning. Based on assayed GHb, selected blood samples were used as examples of high, medium, and low GHb for further studies.

Studies. In the first study, we compared the GHb determined directly from whole blood with that determined from whole blood applied to sample papers. Fifty samples were tested. Circles of paper (14 mm diam) were filled with drops of blood. The blood spots were air-dried at room temperature and then immediately eluted for assay. Next, we measured the effects of applying different amounts of blood (25, 50, 75, and 100 μl) to the sample papers, with samples of low, medium, and high GHb.

To approximate time and temperature conditions potentially faced by sending samples through the mail, we tested the effects of variations in time (3, 6, and 9 days) and temperature (4°C, room temperature, and 37°C) on blood spots stored in tightly sealed plastic bags. Six samples were used for these studies; two were selected for each GHb level.

To determine the influence of blood glucose concentration on GHb measured over time, we added varying amounts of highly concentrated dextrose to one whole-blood sample with a low GHb level, achieving final plasma glucose concentrations of 78–1037 mg/dl (glucose oxidase method with a Beckman glucose analyzer; Fullerton, CA). Blood volume and hematocrit were changed <1% by the added glucose. Spots were prepared for five different plasma glucose concentrations and assayed immediately or stored at room temperature for 3, 6, or 9 days before assay.

Next we tested the effect of removing glucose from the plasma by glucose oxidase or ethanol. Blood with medium GHb level was applied to filter papers with one of three approaches. In the first, filter paper had been dipped into a solution of glucose oxidase (glucose reagent, Beckman) according to the method of Eross et al. (5); in the second, blood was applied to sample papers that were then placed in 100 ml of 70% ethanol for 1 h and air-dried according to the method of Little et al. (6,7); in the third, the sample was spotted and assayed in the usual way. In each case, spots were assayed at day 0 and after 3, 6, or 9 days of storage at room temperature.

To determine whether plasma glucose or intracellular glucose affects GHb measured over time, we compared at days 0, 3, 6, and 9 spots prepared with whole blood with spots prepared with washed red cells from the same sample.

Statistical analysis. Data are means ± SD of percent total hemoglobin eluting as GHb. The paired *t* test was used to compare change in GHb of a single blood sample over variations in time, temperature, and assay method (8). Linear regression was performed according to the Pearson correlation coefficient (8).

TABLE 1
Comparison of GHb in whole-blood assay with GHb in different amounts of blood applied to sample papers

GHb value	GHb (%)				
	Whole-blood assay	25-μl spot	50-μl spot	75-μl spot	100-μl spot
Low	4.3	4.4	4.5	4.4	4.7
Medium	10.3	10.9	10.9	10.8	10.4
High	15.1	15.1	14.5	14.7	14.5

GHb, glycosylated hemoglobin. Each value is 1 sample assayed in duplicate.

TABLE 2
Effect of time and temperature on apparent percentage of GHb

GHb on day 0	GHb at 4°C		GHb at room temperature		GHb at 37°C	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
10.9	11.5	12.6	17.8	16.0	24.5	30.1
14.5	15.4	14.3	20.2	23.0	29.7	34.2
16.3	18.2	19.8	21.9	26.1	36.5	47.0

GHb, glycosylated hemoglobin. Values in percentages. Each value is 1 sample assayed in duplicate.

RESULTS

Very good correlation was found between GHb values measured directly on whole blood and on samples applied to sample paper but immediately assayed ($r = .93$, $P < .001$)

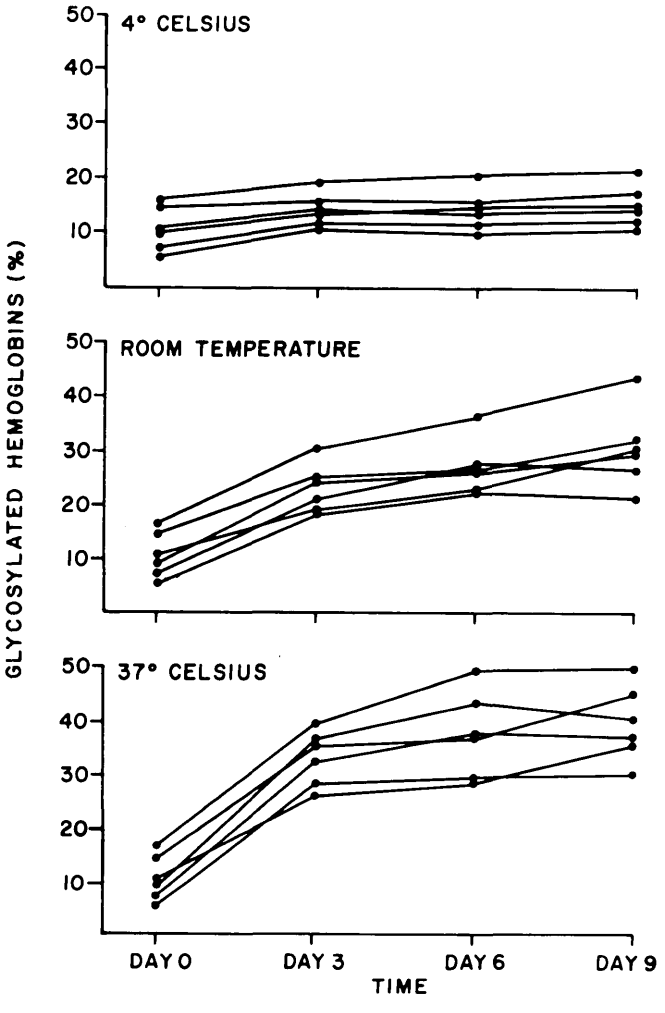

FIG. 2. Effects of sample paper time and temperature in storage on GHb values. Statistically significant increases of apparent GHb values occurred between days 0 and 3 at each temperature ($P < .001$), between days 3 and 6 at room temperature and 37°C ($P < .001$), and between days 6 and 9 at 4°C ($P < .001$) and room temperature ($P < .02$). Other differences not significant.

(Fig. 1). There was a slight but statistically significant trend toward higher GHb when assayed from the sample papers (mean increase 0.64% GHb, $P < .001$). This is indicated by a >0 y-intercept of the data plotted in Fig. 1.

When different-sized drops of sample (25–100 μl) were applied to the paper, GHb results remained within the intra-assay variation of the method (Table 1). Therefore, overloading of the affinity column did not occur even when amounts of blood greater than recommended were applied.

The effects of time and temperature were dramatic. Testing two samples of each blood chosen for their low, medium, or high GHb over 3, 6, or 9 days at three different temperatures, we documented an increase in apparent GHb results with increasing time (Fig. 2). Even over 3 days (judged to be the shortest reliable mailing time) the individual increases ranged from a minimum of 1.3% to a maximum of 27% apparent

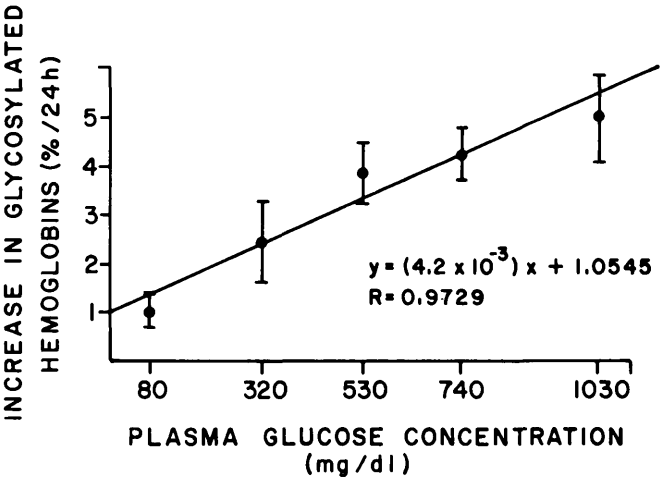

FIG. 3. Effect of plasma glucose concentration on rate of GHb formation. One sample of whole blood was supplemented with glucose and blood spots prepared for 5 different plasma glucose concentrations. The GHb assays were measured on day 0 and after storage for 3, 6, or 9 days at room temperature. Rate of increase in GHb determined by linear regression. Each *point* represents mean ± SD for 4 experiments at indicated glucose concentration. Actual GHb level on day 0 was 5.1 ± 0.3%.

GHb. The rise in apparent GHb over time was clearly temperature dependent. Samples stored at 37°C had mean increases of 24.4, 27.8, and 30.5% GHb at 3, 6, and 9 days, respectively; samples stored at room temperature increased by 12.5, 16.3, and 19.9% GHb, respectively, and samples stored at 4°C increased by only 3.2, 3.5, and 4.7% GHb, respectively. All poststorage values were significantly greater than the baseline value ($P < .001$). Between days 3 and 6, the rise continued significantly ($P < .001$) except at 4°C.

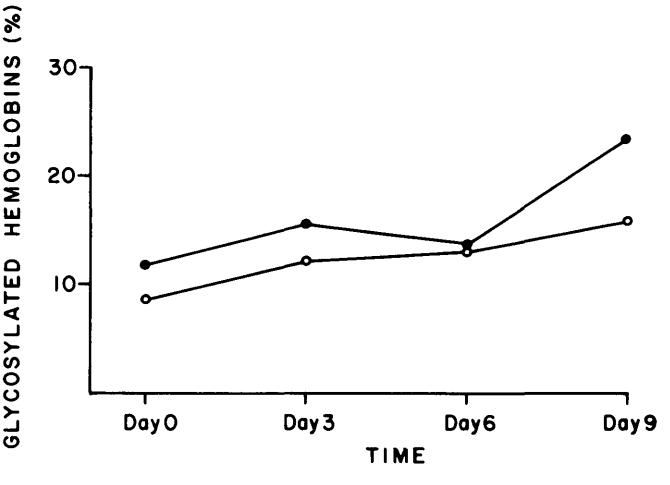

FIG. 4. Effect of glucose oxidase treatment of sample paper on GHb increase over time. Blood from diabetic patient was applied to untreated sample papers (●) and to glucose oxidase–treated sample papers (○). GHb values were measured at day 0 and after storage at room temperature for 3, 6, or 9 days.

Between days 6 and 9, the rise was significant ($P < .02$) except for storage at 37°C, when a plateau was reached at day 6.

We pursued the early time course of this rise in apparent GHb on sample collection papers because the method could be used to deliver samples directly to a laboratory for assay within 1–2 days without the need for venipuncture. Three samples were run on days 0, 1, and 2 (Table 2). Although results were less consistent, apparent GHb was markedly elevated even with 1 day of storage at 37°C and at room temperature and variably elevated when the sample was stored at 4°C.

Variations in plasma glucose concentration did not affect GHb results when assayed immediately. However, apparent GHb did increase over time in proportion to plasma glucose concentration, as artificially adjusted (Fig. 3). Attempts to reduce ambient glucose concentration with glucose oxidase caused lower GHb value at day 0, but the same increase in GHb occurred after storage (Fig. 4). Ethanol-treated spots did not yield reasonable results in this assay. After elution of the blood with NaOH, a precipitate formed during the adjustment of pH necessary for the assay. Finally, spots prepared with washed red cells (i.e., free of plasma) had the same increase in apparent GHb after storage: from 5.6% at day 0 to 11.2 and 13.2% after 3 and 9 days of storage at room temperature.

DISCUSSION

If the sample-paper collection method were reliable, it would be possible for patients to send samples for GHb assay to the physician's office or laboratory between visits. This would allow the physician to know when control was deteriorating without a patient visit. It would also be convenient for the physician to have a recent GHb result to discuss at a visit without the patient having to arrange for venipuncture several days earlier. In our study, however, the sample-paper method proved unreliable.

The GHb assay by affinity chromatography measures all glycosylated residues occurring on HbA_{1c}, HbA_{1b}, HbA_{1a}, or elsewhere (4,9–13). It is a relatively hardy method by most criteria, less susceptible to variations in column temperature, pH, and other laboratory variables than ion-exchange columns (11–13). The presence of the labile aldimine form of GHb (14) seems to have little or no effect on the quantitation of GHb by this method (11,13). Storing samples of whole blood at 4°C for as long as 2 mo does not significantly alter GHb values determined by affinity chromatography (13). At 20°C, the affinity method shows sample stability for 1 wk (15). Glucose concentration in the physiologic range does not affect GHb results. Little et al. (16) showed a significant increase in GHb only when plasma glucose concentrations were >672 mg/dl and after storage of blood samples at room temperature for 14 days.

It has been shown that formation of HbA_{1c} increases with time, glucose concentration, and temperature during incubation of intact human erythrocytes in vitro (17,18). Although the material causing the rise in GHb has not been identified in our study, it may represent additional glycosylation of hemoglobin during storage. Goldstein et al. (14) had similar results for glycosylated proteins measured by a colorimetric assay in whole blood spotted on filter paper. The increase in whole-blood glycosylated proteins was proportional to the duration of storage and to the concentration of glucose in the blood spots. However, these authors succeeded in preventing in vitro glycosylation of blood proteins by washing the filter-paper spots in alcohol (6,7). For the same assay, treatment of filter papers with glucose oxidase prevents in situ glycosylation of blood (5). In our study, glucose removal with ethanol was not compatible with the affinity assay and glucose oxidase only partly inhibited the in vitro glycosylation. Because we found that a similar GHb increase occurs on spots prepared with washed red cells and with whole blood, removal of free glucose may not be sufficient to inhibit in situ glycosylation.

In conclusion, although affinity chromatography has been well established as a reliable assay of glycosylated hemoglobins, our findings suggest that blood samples may not be stored on paper, at least by available techniques, before assay. Further studies are necessary to allow the successful clinical application of the sample-paper approach.

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