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Effect of Sample Instability on Glycohemoglobin (HbA₁) Measured by Cation-Exchange Chromatography

Marcia Simon and J. David Hoover

We compared results for glycohemoglobin obtained from fresh whole blood or separated erythrocyte samples with results obtained after storage. We judged the storage condition to be acceptable ("stable") if the glycohemoglobin results after storage were within the 95 confidence interval (± SD) of the results obtained for the specimens on the day of venipuncture. Hemolysates can validly be stored for five months at −70 °C. Whole-blood samples stored at 4 °C remain stable for four days; whole blood treated with heparin or EDTA (but not oxalate) is stable for seven days. At 30 °C, whole blood or erythrocytes from some donors are stable for one day, but after two days and seven days, results are frequently higher. We confirmed previous findings that the separated erythrocytes can be stored at −20 °C for at least seven days. In addition, we compared the elution profiles for stored samples showing increased values.

Addition Keyphrases: *sample handling · variation, source of · preparation of control materials*

The assay for glycosylated hemoglobin (glycohemoglobin, HbA₁, fast Hb) is a new laboratory test with good potential for facilitating an improvement in the clinical management

of patients with diabetes mellitus (*1–3*). Several techniques including colorimetry (*4*), isoelectric focusing (*5*), and RIA (*6*) have been described for glycohemoglobin quantitation, but more popular current methods are based on cation-exchange chromatography. The many published methods differ markedly in resolution and in suitability for the routine clinical laboratory (*7–9*). While some methods measure only HbA₁c, which is characterized by a covalent linkage of 1-amino-1-deoxyfructose to the N-terminal valines of the beta chains, most chromatographic methods measure the entire "fast" fraction, believed to include HbA₁a and HbA₁b (*7*). The source and function of the other minor hemoglobins are not yet fully understood (*2*).

The stability of the hemoglobins is fundamental to the preparation of quality-control materials for monitoring method performance and to the interpretation of results for patients' specimens after storage.

Few quantitative data have been published regarding the effects of sample instability on glycohemoglobin results. The present study was designed to evaluate storage conditions such as short-term delays encountered by specimens in reaching the laboratory and longer storage such as would be involved for quality-control materials used to monitor test performance and interlaboratory comparisons.

Materials and Methods

Glycohemoglobin assay. A description of the analytical method and its validation appears elsewhere (*7*). Briefly, we hemolyzed unwashed erythrocytes with carbon tetrachloride.

Table 1. Monthly Means for Glycohemoglobin in Hemolysates Stored at −70 °C (% of Total Hemoglobin)

	Months of storage				
	1	2	3	4	5
Normal donor	7.1	7.1	7.2	7.0	7.3
Diabetic donor	11.8	11.7	11.8	11.4	12.2
Pooled samples	15.7	15.7	15.8	16.0	15.2

After removing cell debris by centrifugation, we chromatographed the hemolysates on small disposable columns containing BioRex-70 (200–400 mesh) previously equilibrated with cyanide–phosphate buffers. After elution of the fast chromatographic fraction (containing HbA_{1a+b} and HbA_{1c} as separate peaks), we compared the absorbance at 416 nm of the fast fraction with the absorbance of the diluted total hemolysate to determine the percentage of glycohemoglobin. We prepared all buffers and eluted the columns at a temperature of 19–21 °C. We monitored the eluting strength of the buffers by measuring resistance and pH to three significant digits. The between-run imprecision (CV) for a normal pool (mean glycohemoglobin, 7.5% of total hemoglobin) was 3.4% and of an above-normal pool (mean 12.3%) was 5.1% over the period of stability testing.

Samples. Heparin-, EDTA-, or oxalate-treated whole blood was drawn from adult volunteers so as to completely fill each collection tube. Erythrocytes were removed from aliquots of the specimens within 1 h of collection and the glycohemoglobin was quantitated for "day 0" results. Separate aliquots of the erythrocytes were stored at 30 or −20 °C and analyzed on days four and seven. Aliquots of whole blood were stored at 4 and 30 °C for two, four, and seven days. Each group was distributed into several batches, to randomize the effect of between-run variations. For precision studies, we prepared hemolysates of blood from a normal adult donor, a diabetic adult donor, and patients' specimens showing increased glycohemoglobin results. These hemolysates were aliquoted and stored at −70 °C.

Results

*Hemolysates.* Table 1 shows some monthly average glycohemoglobin results from hemolysates stored at −70 °C and analyzed over the last 18 months. The normal and diabetic donor pools showed slight increases (<1 SD) by the fifth month of use. A significant increase was not observed in the specimen pool until the sixth month, when the mean rose to 16.6% (>1 SD). These results are consistent with our observation that hemolysate pools are usable for about five months if stored at −70 °C.

Whole blood. Figure 1 shows the comparison of glycohemoglobin results obtained from fresh samples with results obtained after storing whole blood for two, four, and seven days at 4 °C. All results from heparin- or EDTA-treated whole blood were within the 95% confidence interval around the results from fresh samples (±2 SD). In contrast, results from most of the oxalated whole blood samples were higher by the seventh day of storage at 4 °C. Hemolysis was more common in this group of specimens.

The effects of 30 °C storage of whole blood on glycohemoglobin results are shown in Figure 2. All of the results obtained from samples stored for one day were within the 95% confidence interval around the results from fresh samples. By day two, however, eight of 20 results exceeded the precision limits, and at day seven, 22 of 25 results were significantly increased.

Erythrocytes. We also investigated the stability of erythrocytes stored for various intervals at 30 and −20 °C (Figure
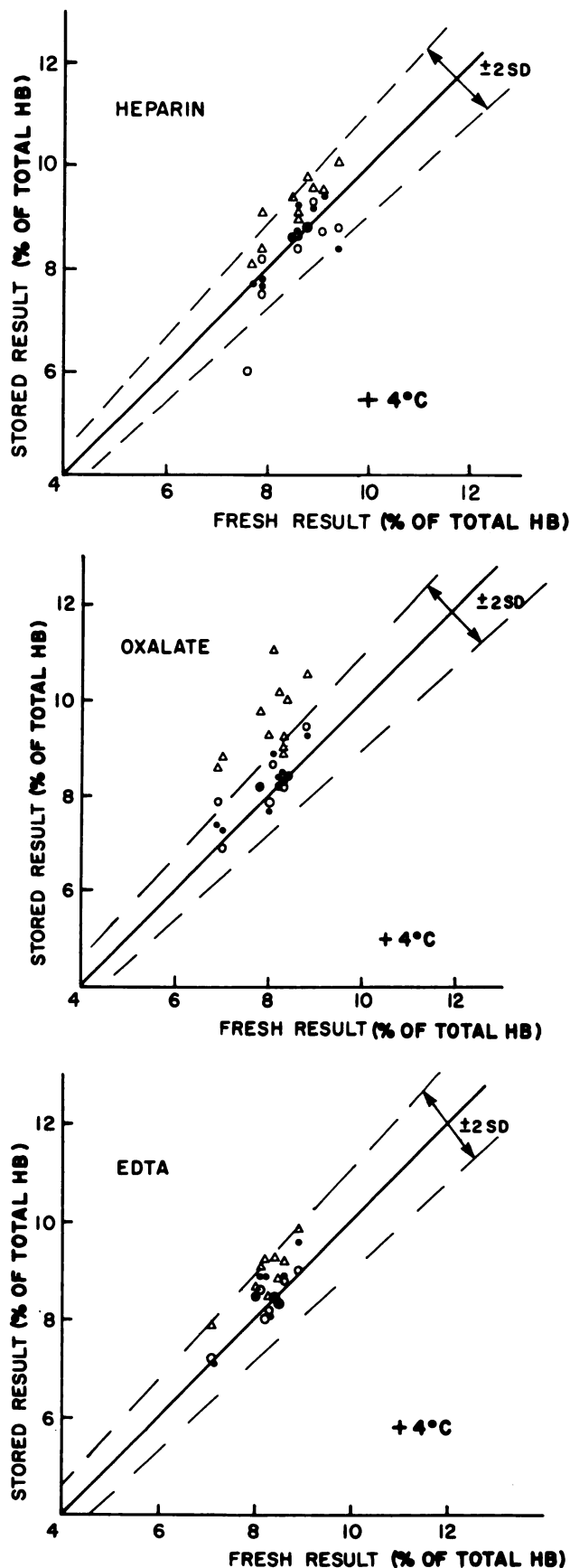
Fig. 1. Glycohemoglobin results (as percent of total hemoglobin) in freshly separated erythrocytes and in cells separated from heparin-, oxalate-, or EDTA-treated whole blood stored at 4 °C

Solid line shows, for comparison, the line of complete agreement ($x = y$). Storage days 2 (O), 4 (●), and 7 (△)
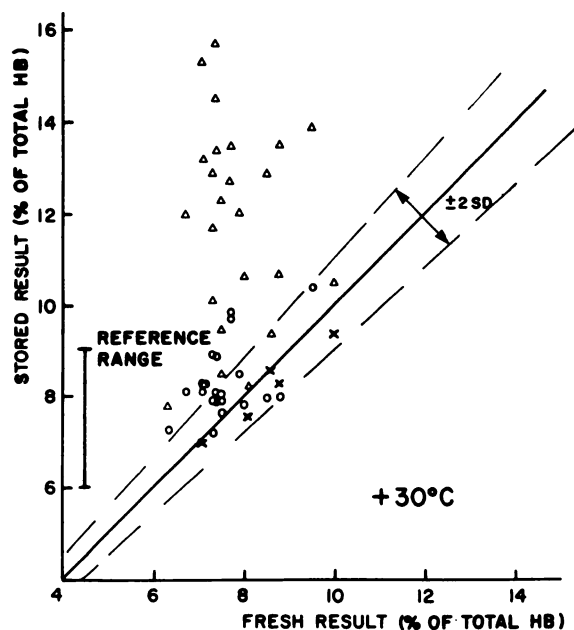
Fig. 2. Glycohemoglobin results (as percent of total hemoglobin) in freshly separated erythrocytes and in cells separated from heparin-, oxalate-, or EDTA-treated whole blood stored at 30 °C

Solid line shows, for comparison, the line of complete agreement ($x = y$). Storage days 1 (X), 2 (O), and 7 (△)

3). As we previously reported (7), the results for glycohemoglobin in cells stored frozen for up to seven days did not differ significantly from the results for the same specimens before storage. In contrast, results for half the erythrocyte samples were higher by day four of storage at 30 °C, and 12 of 15 were increased at day seven.

Elution profile. We examined the profile of hemoglobin eluted from the columns. Hemolysates were prepared from erythrocytes stored for seven days at 4, 30, or −20 °C. Figure 4 shows the separation of HbA_{1a+b} from HbA_{1c} and the appearance of extra peaks formed under some storage conditions. The position of the HbA_{1c} peak was confirmed by eluting a purified HbA_{1c} standard described earlier (7). A slight increase in the HbA_{1a+b} peak and a small late-eluting peak at 8 mL are seen in the hemolysate from frozen erythrocytes. The first 5 mL contains the fast fraction in the routine glycohemoglobin method, and late peaks are not included in the measurement. Therefore, the profile observed is consistent with our previous report that glycohemoglobin results after frozen storage of cells for seven days were not significantly different from the results obtained for freshly drawn specimens. Large peaks co-eluting with HbA_{1a+b} and HbA_{1c} are found from cells stored at 4 or 30 °C. The late-eluting peak is particularly apparent in some samples of cells stored at room temperature. By subjecting the stored specimens showing increased glycohemoglobin results to isoelectric focusing, we have shown previously that these increased peaks do *not* represent HbA_{1c} (5). In these cases, the HbA_{1c} band in the stored samples was quantitatively equivalent to the band observed in fresh samples. In the stored specimens showing increased glycohemoglobin, isoelectric focusing showed additional bands with pI values similar to the previously described pI values for hemoglobin breakdown products (10). The patterns observed are consistent with the higher results for erythrocytes stored at 4 and 30 °C.

Discussion

Hemolysates from some blood samples show increased results for glycohemoglobin after storage of the hemolysate for
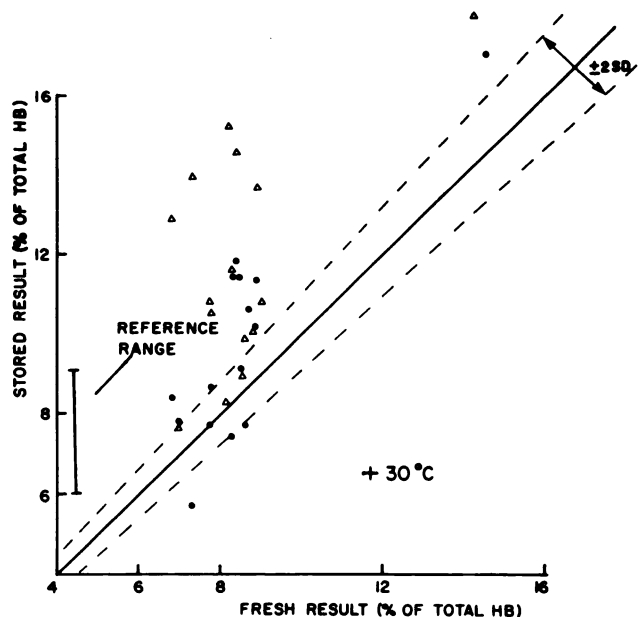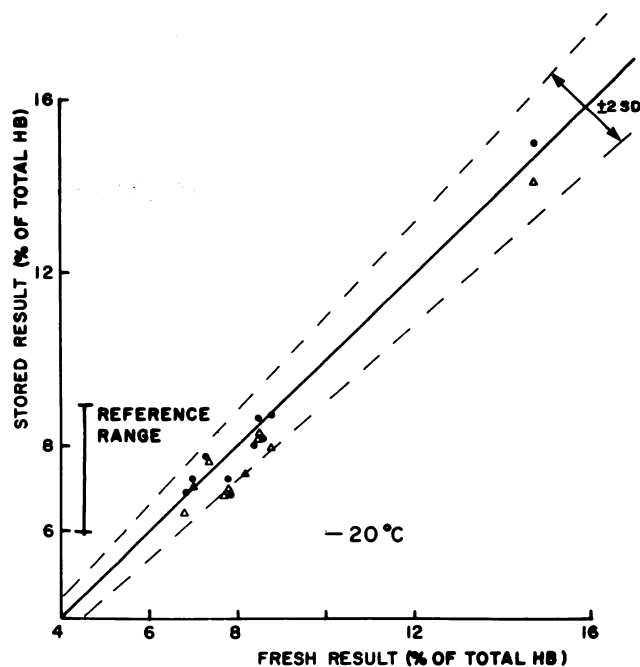

Fig. 3. Glycohemoglobin results (as percent of total hemoglobin) in freshly separated erythrocytes and in cells stored at −20 and 30 °C for 4 days (●) and 7 days (△)

Solid lines show, for comparison, the line of complete agreement ($x = y$)

10 days at −20 °C (7, 11). Other investigators have reported that hemolysates were stable for an unspecified time when stored at −70 °C (12, 13). The results shown in Table 1 suggest that storage at −70 °C for five months is acceptable. We observed a steady increase in results from hemolysates stored at −70 °C for longer than five months (data not shown), although hemolysates are reportedly stable for at least a year at −90 °C (11). We believe that hemolysates stored at −70 °C are useful as quality-control pools and could be suitable for interlaboratory comparisons.

However, hemolysate cannot always be conveniently prepared promptly after a blood specimen is obtained, and so we sought other conditions under which a specimen could be validly transported. Unfortunately, storage of whole blood at 30 °C for longer than a day results in increased glycohemoglobin values for many normal specimens (Figure 2). Storage
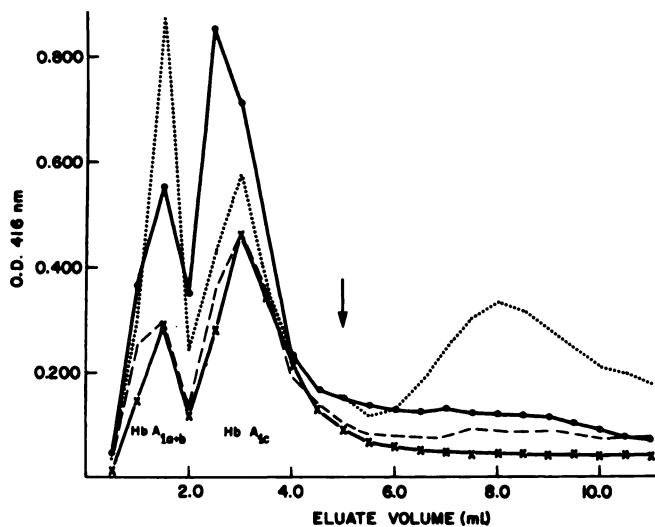
Fig. 4. Profiles (A_{416nm}) of hemoglobins eluted from the ion-exchange columns for glycohemoglobin analysis

Hemolysates prepared from freshly separated erythrocytes (X) and after seven-day storage of the separated cells at 30 (· · ·), 4 (●), and −20 °C (- -). The arrow indicates that the first 5 mL of elute is collected for routine analysis of glycohemoglobin

at 4 °C for as long as seven days appears to be acceptable if the anticoagulant is heparin or EDTA, but not oxalate (Figure 1). A disadvantage is that glycohemoglobin results from EDTA-treated blood depend greatly on the amount of blood in the collection tube (9).

Even under the more extreme conditions, such as storage of whole blood at 30 °C for seven days, some samples still gave unchanged results. This may explain discrepancies with some previous reports, because most investigators have examined only a few samples. Our observations are in contrast to the two samples tested by Baron et al. (9), who found *decreased* results after two days for heparin, fluoride-, or citrate-treated blood but 14-day stability for EDTA-treated blood stored at 4 °C. Others (8, 9, 14, 15) advise storage of whole blood at 4 °C for one to 30 days, but few supporting data have been published.

We have been unable to find a measurement to assess whether shipped samples have been maintained at 4 °C at all times during transit. Even a tedious observation of each individual column-elution pattern will not reveal whether a high result is valid or is secondary to poor sample stability because of the increases observed in the HbA_{1a+b} and HbA_{1c} peaks (Figure 4). Therefore, frozen storage is desirable.

We avoided frozen whole blood in investigations because we observed increased glycohemoglobin in heparin-, oxalate-, or EDTA-treated whole blood after storage at −20 °C for seven days (7), in contrast to observations by other investigators (15). Frozen whole blood is also unsuitable, as indicated in a report that plasma included in the lysed whole blood after frozen storage contains substances that interfere with absorbance measurements (16).

Figure 3 shows that the cells can be stored at −20 °C for seven days without significant alteration in the glycohemoglobin result. We have not studied cells stored longer than this, and these results should not be extrapolated to longer times.

We find that maintaining frozen storage is simpler and more reliable than trying to maintain 4 °C during transit.

Many of our findings probably can be confirmed by cation-exchange methods other than the one we used. As shown in Figure 4, some of the hemoglobin degradation products from poorly stored specimens are eluted almost exactly coincident with the HbA_1 peaks. The late-eluting peak may be HbA_{1d} or Hb_{1e}, which were observed by early investigators (17). The inclusion of this peak in the "fast fraction" measurement depends on the exact chromatographic conditions used; it was excluded in the present method.

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