with none of the four kits compared well with each other for ferritin concentrations >300 μg/L. Thus, the problem of monitoring iron overload with serum ferritin measurements may be further complicated by discrepancies among commercial kits.

References

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Effects of Whole Blood Storage on Results for Glycosylated Hemoglobin as Measured by Ion-Exchange Chromatography, Affinity Chromatography, and Colorimetry
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After storage of whole blood at either 4 or 20 °C, results for glycosylated hemoglobin by ion-exchange chromatography ("high-performance" liquid and mini-column chromatography), thiobarbituric acid colorimetry, and affinity chromatography were compared. At 4 °C, all methods gave acceptable results for samples stored for as long as a week. At 20 °C, the colorimetric and affinity methods also showed sample stability for a week or more. The ion-exchange methods were associated with a marked increase in values for glycosylated hemoglobin after a few days of storage. Evidently, care in details of sample collection and handling is especially important for ion-exchange methods, and the colorimetric and affinity methods have advantages over ion exchange in situations where long delays between sample collection and assay are unavoidable.

Additional Keyphrases: screening • diabetes

Glycosylated hemoglobin assay is a clinically useful index of long-term blood glucose control in diabetes mellitus (1–3). Several assay methods have been described, but little attention has been given to effects of sample handling and storage before processing and analysis, although these factors may be of great importance for the clinical laboratory (4, 5).

Here we present data on the stability of glycosylated hemoglobin in whole blood stored for as long as 15 days before hemolysate preparation and analysis for glycosylated hemoglobin. We compare results obtained by several different assay methods: ion-exchange chromatography ("high-performance" liquid (HPLC-IE) and mini-column chromatography), thiobarbituric acid (TBA) colorimetry, and affinity chromatography.

Materials and Methods
Blood samples. All specimens were obtained by venipuncture, from five Type I (insulin-dependent) diabetic patients and from five non-diabetic subjects. All diabetic patients were being treated with two daily injections of insulin. Blood specimens were collected into EDTA-containing evacuated blood-collection tubes and promptly refrigerated (4 °C). Within 2 h of collection, all specimens were divided and stored for various periods of time up to 15 days at either 4 or 20 °C in capped 12 × 75 mm tubes.

Preparation of hemolysates. At designated intervals, whole-blood samples were mixed thoroughly by inversion and aliquots removed for preparation of hemolysates as previously described (6). Briefly: for each specimen, plasma and leukocytes were removed after centrifugation (800 × g, 10 min). Erythrocytes were washed three times with five volumes of isotonic saline and incubated in 12 volumes of

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isotonic saline at 37 °C for 5 h. Erythrocytes were then lysed in distilled water (threefold dilution). An equal volume of carbon tetrachloride was added, and the preparation was mixed and refrigerated at 4 °C overnight. After centrifugation (27 000 × g, 30 min, 4 °C), the aqueous supernate was stored at −70 °C until analysis in batch by each method.

**Assay methods.** Four different assays were performed on each hemolysate sample.

1. For affinity chromatography we used a commercially available kit (Glycosylated Hemoglobin Clinovative Diagnostic Kit; Pierce Chemical Co., Rockford, IL 61105) according to the product insert instructions.

2. Colorimetry was by the improved thiobarbituric acid (TBA) method of Parker et al. (7), with fructose as the standard.

3. Mini-column ion-exchange chromatography (Isolab, Inc., Akron, OH 44321) was used to measure total "fast" glycosylated hemoglobin, designated HbA₁ (HbA₁ = HbA₁a+b+c) according to the product insert instructions. Assay temperature was controlled by use of a water jacket and constant temperature bath set at 22 °C.

4. The automatically controlled "high-performance" liquid chromatography–ion-exchange method (HPLC-IE) of Goldstein et al. (6) was used to measure the percentages of HbA₁a and HbA₁c.

**Analysis of data.** For each assay method, effects of storage on glycosylated hemoglobin values are presented as percent change from day 0 (means ± SEM). A mean value was considered to differ significantly from that at day 0 if it was outside the acceptable range for the particular assay (calculated as 2 SD beyond the mean of a pooled quality-control sample run repetitively and converted to percent of baseline value). For example, a 100% increase in the presented mean value compared to day 0 represents a doubling of the mean absolute value.

**Results and Discussion**

Effective clinical use of glycosylated hemoglobin requires reproducible and accurate test results (6). If hemolysates are promptly prepared after sample collection and stored at −70 °C, glycosylated hemoglobin values are well reproducible after many months of storage, regardless of the assay method (4, 6, 8). However, in the usual clinical setting, there are often delays before blood samples can be processed, and −70 °C storage conditions are often not available.

Figure 1 shows the effects on glycosylated hemoglobin of storing whole blood at 4 or 20 °C for up to 15 days. Storage of whole blood at 4 °C for as long as a week was associated with acceptable assay results with each of the methods studied. Our findings for 4 °C storage are consistent with the reports of Simon and Hoover (4) for ion-exchange mini-columns and of Jury et al. (5) for HPLC-IE.

At 20 °C our results (Figure 1) showed increases in HbA₁ relative to day 0 for both ion-exchange methods, values being as much as 50% greater after one week of storage. Samples from non-diabetic subjects showed consistently greater percentage increase in values from day 0 than did samples from diabetic patients, although the absolute increases were similar in both groups (e.g., HbA₁ increase from 5 to 6% in control and 10 to 11% in diabetics). Studies by others have shown similar changes (4, 9). Our results with ion-exchange mini-columns differ from those reported by both Walinder et al. (10), who found only a modest increase in HbA₁ values after whole blood storage at 22 °C for 15 days, and Hammon et al. (11), who found a decrease in HbA₁ values for diabetic but not control samples stored at room temperature for 14 days. We cannot explain these differences in results.

![Fig. 1. Effect of whole-blood storage on values for glycosylated hemoglobin as measured by (top to bottom): the affinity method; TBA colorimetric method; the mini-column ion exchange method, with measurement of HbA₁; the HPLC-IE method, with measurement of HbA₁; and the HPLC-IE method, with measurement of HbA₁c. Points and vertical bars represent the mean (± SEM) percent change in values for glycosylated hemoglobin, relative to day 0, for five non-diabetic and five diabetic samples. Shaded area: 95% confidence limits for the assay base on repetitive quality-control sample analysis.](image-url)
Examination of the minor hemoglobin fractions by use of the HPLC-IE method showed that the large increase in percent HbA at 20 °C was mainly ascribable to an increase in the hemoglobin A_{1a+b} fraction, with only a small increase in hemoglobin A_{1c} until eight days of storage (4).

Samples assayed by affinity chromatography and TBA colorimetry showed excellent stability at both 4 and 20 °C, although after 10 days of storage, affinity-assayed samples stored at 20 °C showed higher results. In other experiments on the affinity method (Little et al., 1983, this issue) we found that values for glycosylated hemoglobin are not increased with storage of whole blood at 20 °C for as long as three weeks if hemolysates are prepared directly from unwashed packed cells without saline wash or incubation.

We conclude that great care must be taken with details of sample collection and handling for ion-exchange chromatography assay methods, particularly those that measure only HbA_. Each laboratory should validate storage conditions for their particular assay method and sample handling procedures. Our results suggest that with respect to sample handling and stability characteristics, the affinity chromatography and TBA colorimetry methods may have advantages over ion-exchange chromatographic methods, especially in situations where samples cannot be refrigerated shortly after collection or delays between sample collection and refrigeration for transport to the laboratory may occur.

Our data show that for all of the methods tested, sample stability was excellent with 4 °C storage conditions for at least eight days. For the laboratory that carries out relatively few glycosylated hemoglobin assays per day, whole-blood samples can be stored for at least several days at 4 °C and assayed concurrently.

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References

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Boehringer Immunoinhibition Procedure for Creatine Kinase-MB Evaluated and Compared with Column Ion-exchange Chromatography

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In determination of creatine kinase isoenzyme MB (CK-MB), the Boehringer immunoinhibition method gives a high and variable blank activity as compared with column-chromatography. Thus a correction must be applied. Furthermore, a second correction of 1% of total creatine kinase activity is necessary to compensate for nonspecific creatine phosphate-dependent activity. As a consequence, two immunoinhibition determinations—one for CK-MB and one for blank activity—and a determination of total creatine kinase are required. Use of the manufacturer's diagnostic criteria, on the basis of which suspected myocardial infarction is confirmed or eliminated, leads to a high frequency of false-negative conclusions.

Additional Keyphrases: myocardial infarction · isoenzymes · heart disease · "kit" methods

Determination of the MB isoenzyme of creatine kinase (CK; EC 2.7.3.2) is generally accepted as the best biochemical diagnostic test for acute myocardial infarction, and its ion-exchange (column) chromatography is firmly established as a method meeting the criteria for a clinically useful method (1, 2).

Recently, much attention has been paid to immunoinhibition methods for determination of CK-MB. In a previous communication (2) we presented our experience with the immunologic Merck MB kit, and clinical evaluations of the "Cardiozyme" immunoinhibition (Dade) and the "Isomune" kit (Roche Diagnostics) have been published (4-7). Apparently, especially at low activities of CK-MB, these immunological methods still lack the necessary accuracy.

In view of our interests in an accurate and rapid technique for CK-MB determination, we compared ion-exchange chromatography as used in our laboratory with the Boehringer immunoinhibition (B.M.C.) kit (no. 300691; Boehringer Mannheim GmbH, 68 Mannheim 32, F.R.G.).

Materials and Methods

Sample handling and ion-exchange column chromatography were as described previously (2, 3), except that the