Critical Factors in the Chromatographic Measurement of Glycohemoglobin (HbA_1)

M. SIMON AND J. EISSLER

SUMMARY

Measurement of glycohemoglobin has been proposed as a criterion for the management of diabetes mellitus. We evaluated various conditions critical to the accuracy and precision of the cation-exchange method.

Tolerance limits for each variable were defined as follows: phosphate-eluding buffer (0.06 ± 0.005 mol/L ph 6.70–6.72), column temperature (19–21°C), and resin equilibration (to phosphate buffer, 0.07 mol/L, pH 6.70 ± 0.01). Hemoglobin absorbance measured in the Sorét region (approximately 416 nm) of the first chromatographic fraction divided by that of the total hemolysate provided the most accurate and precise result. Overall between-run precision expressed as coefficient of variation (CV, in percent) of normal and diabetic pools was 4.8% and 5.1%, respectively. When purified HbA_{1c} was added to hemolysates, recovery was 90–95%. Results were linear to at least 18% glycohemoglobin. Hemoglobin F (HbF) interfered with the method, whereas HbC and HbS did not. Red cells could be stored frozen for at least 6 days, thus easing transport of outpatient samples. A reference range of 6.0–8.8% glycohemoglobin was established from 85 nondiabetic adults (ages 23–65 yr). In a clinical study, only 4 of 13 treated diabetic patients believed to be in good control showed glycohemoglobin results within the normal range. All of the 19 treated diabetics in fair or poor control showed glycohemoglobin results greater than 10% of total Hb, ie., well above the normal range. DIABETES 29:467–474, June 1980.

It has been shown recently that the hyperglycemia of diabetes mellitus may be reflected in the formation of glycohemoglobin (glycosylated or fast hemoglobin, HbA_1). Glycosylation of hemoglobin (Hb) appears to occur concurrently with glycosylation of other proteins, and these changes may be responsible for nephropathy, retinopathy, and other complications.[1] Thus, measurement of glycohemoglobin may prove an important criterion for the clinical management of diabetic patients.[2] Several methods have been proposed for measurement of glycohemoglobin and most of these are based on cation-exchange separation of hemoglobin fractions.[3–5] Published descriptions have not included standards or procedures for preparing quality control pools. Lacking those conventional mechanisms for controlling accuracy and precision, the likelihood of long-term drift in the assay results is high.[6] Such assay drift would preclude long-term studies of the relationship of glycohemoglobin to the onset and progression of chronic complications.

HbA_{1c} was purified in the present study and was used with stabilized quality control pools to determine the critical conditions in a cation-exchange method for measurement of glycohemoglobin. While "glycohemoglobin" may be used to indicate *all* carbohydrate-containing hemoglobins,[10,11] glycohemoglobin here refers to the fast eluting chromatographic fraction (HbA_1), including HbA_{1a+b+c} and HbF (if present). Other chromatographic fractions comprised HbA_0 (the major adult hemoglobin $\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$).

MATERIALS AND METHODS*

Purification of standard. HbA_{1c} was purified from the red cells of a diabetic donor using preparative methods;[12] about 67% of the original HbA_{1c} was recovered. The homogeneity of this preparation was examined using an isoelectric-focusing technique designed to separate the HbA_1 components.[13] Focusing yielded a single band of HbA_{1c}.

Equilibration of resin. The cation-exchange resin employed in this study was obtained in several lots from Bio-Rad Laboratories (Richmond, California) as Bio-Rex-70, 200–400 mesh. It was washed once with distilled water by

From the Research Department, Bio-Science Laboratories, Van Nuys, California

Address reprint requests to M. Simon, Ph D., Research Department, Bio-Science Laboratories, 7600 Tyrone Avenue, Van Nuys, California 91405.

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* Accurate preparation of the buffers and adequate chromatographic performance require ambient temperatures of 19–21°C. This temperature is maintained by placing the columns in an LKB Combi-Cold, or equivalent, cabinet.

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stirring the resin using a glass rod with 2 vol of water. After allowing 15 min for settling, the fines were removed by pouring off the supernatant. The resin was converted from the sodium form by adding glacial phosphoric acid to a 1:3 (vol/vol) aqueous slurry of the resin until the pH reached 6.7. The resin was then washed twice with 2 vol of 0.5M buffer, pH 6.70 ± 0.02. The resistance of this buffer was found to be 30–35 Ω using a conductivity bridge (Yellow Springs Instrument, cell constant = 1.0). The resin was equilibrated against each batch with 0.05M sodium phosphate in 0.01M KCN (pH 6.70–6.72, resistance 220–240 Ω) until the pH of the supernatant matched that of the buffer. The resin was then equilibrated with 4-vol batches of 0.07M sodium phosphate in 0.01M KCN (pH 6.70–6.72, resistance 172–182 Ω) until the resistance and the pH of the supernatant exactly matched the buffer.

Routine method. One milliliter of red cells [separated from heparin or ethylenediaminetetraacetate (EDTA) plasma by centrifugation] was lysed by shaking with 2 ml of distilled water and 3 ml of carbon tetrachloride. After centrifuging 15 min at 1000 *g*, the upper phase (hemolysate) was removed fo use.

Disposable polyethylene transfer pipets (Scientific Products no. 5214-10, 6-in. size) were converted into columns by cutting off the top of the pipet bulbs to form reservoirs and tamping small cotton plugs into the tips.

Resin, batch-equilibrated with the cyanide-containing phosphate buffers just described, was slurry packed into disposable polyethylene transfer pipets to a bed height of 3 in.

Hemolysate (20 μl) was applied to the surface of the resin bed, and 200 μl eluting buffer (0.06M sodium phosphate containing 0.01M KCN, pH 6.70–6.72, resistance = 230–210 Ω) was used to rinse the sample into the bed. Eluting buffer (5 ml) was added to the reservoir and the entire eluate was collected (fast fraction). The flow rate was roughly 0.05 ml/min. We calculated the percent fast hemoglobin by dividing the absorbance of the fast fraction at 416 nm by that of 1:2000 aqueous dilution of the hemolysate. The performance of the method was monitored by including the purified A_{1c} standard as well as the nondiabetic and diabetic hemolysates (stored at −70°C and used as quality control pools) in each batch of samples.

Clinical range studies. Heparin, EDTA, or oxalated whole blood was obtained from 85 consenting adults (ages 23–65 yr) presenting no history of hyperglycemia. Thirty-four diabetics (ages 26–33 yr) attending an outpatient clinic were evaluated to be in good, fair, or poor control according to criteria used by Tze.[14]

RESULTS

Optimization of assay variables. The effects of chromatographic conditions on the separation of glycohemoglobin from HbA_0 were examined by comparing purified A_{1c} recovery and pool results when each parameter was varied independently. Under conditions where there was little resolution of the individual A_1 hemoglobins, separate bands could not be seen as the column was eluting and the main A_0 hemoglobin band merged with the fast eluting hemoglobins: this observation is termed A_0 breakthrough in this report. In the first experiment, shown in Figure 1, the resin was equilibrated to pH 6.72 with phosphate buffer (0.05M containing

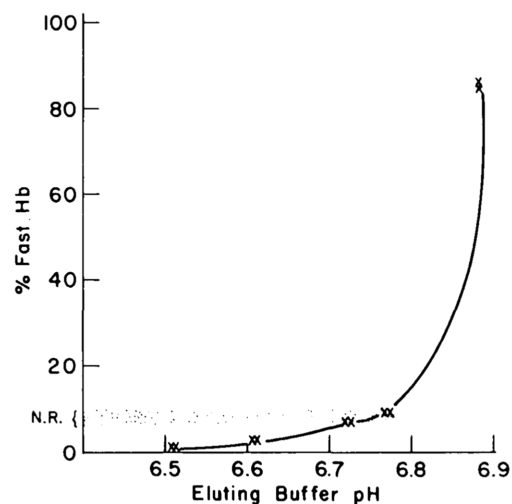

FIGURE 1. Effect of elution buffer pH on the percent hemoglobin recovered in the glycohemoglobin fraction using 20 μl of a nondiabetic hemolysate. All buffers were 0.05M sodium phosphate with 0.01M KCN. The resin was equilibrated to the same phosphate buffer (pH 6.70 ± 0.02). The normal range is indicated by the shaded area (N.R.).

0.01M potassium cyanide) and eluting buffers of pH 6.2 to pH 8.5 were examined using a nondiabetic hemolysate pool. In accord with other reports,[3] pH 6.72 buffer provided good recovery of HbA_{1c} with little HbA_0 contamination. The elution depended highly on pH, and the pH tolerance of the buffer was less than 0.05 pH units.

In Figure 2 are the results of a nondiabetic hemolysate chromatographed at several different eluting buffer ionic strengths with a constant pH of 6.70 and 0.01M KCN. The elution of hemoglobin in the first 5 ml depended highly on the molarity of the phosphate buffer used. At 0.025M, little hemoglobin eluted, while at 0.1–0.15M almost *all* hemoglobin fractions eluted in the first 5 ml of buffer.

The results of experiments using purified HbA_{1c} and a nondiabetic hemolysate in which the ionic strength was varied

FIGURE 2. Effect of elution buffer ionic strength on the percent hemoglobin recovered in the glycohemoglobin fraction using 20 μl of a nondiabetic hemolysate. All buffers were sodium phosphate, pH 6.70–6.72, with KCN. The resin was equilibrated to 0.05M sodium phosphate containing 0.01M KCN, pH 6.70–6.72. The normal range is indicated by the shaded area (N.R.).

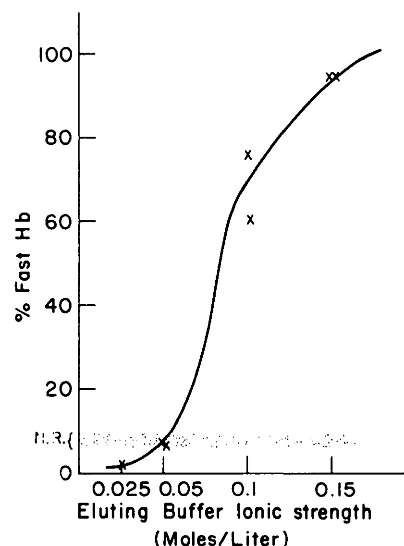

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TABLE 1
Effect of molarity of phosphate buffers (pH 6.70) on elution of the glycohemoglobin fraction and purified HbA_{1c}

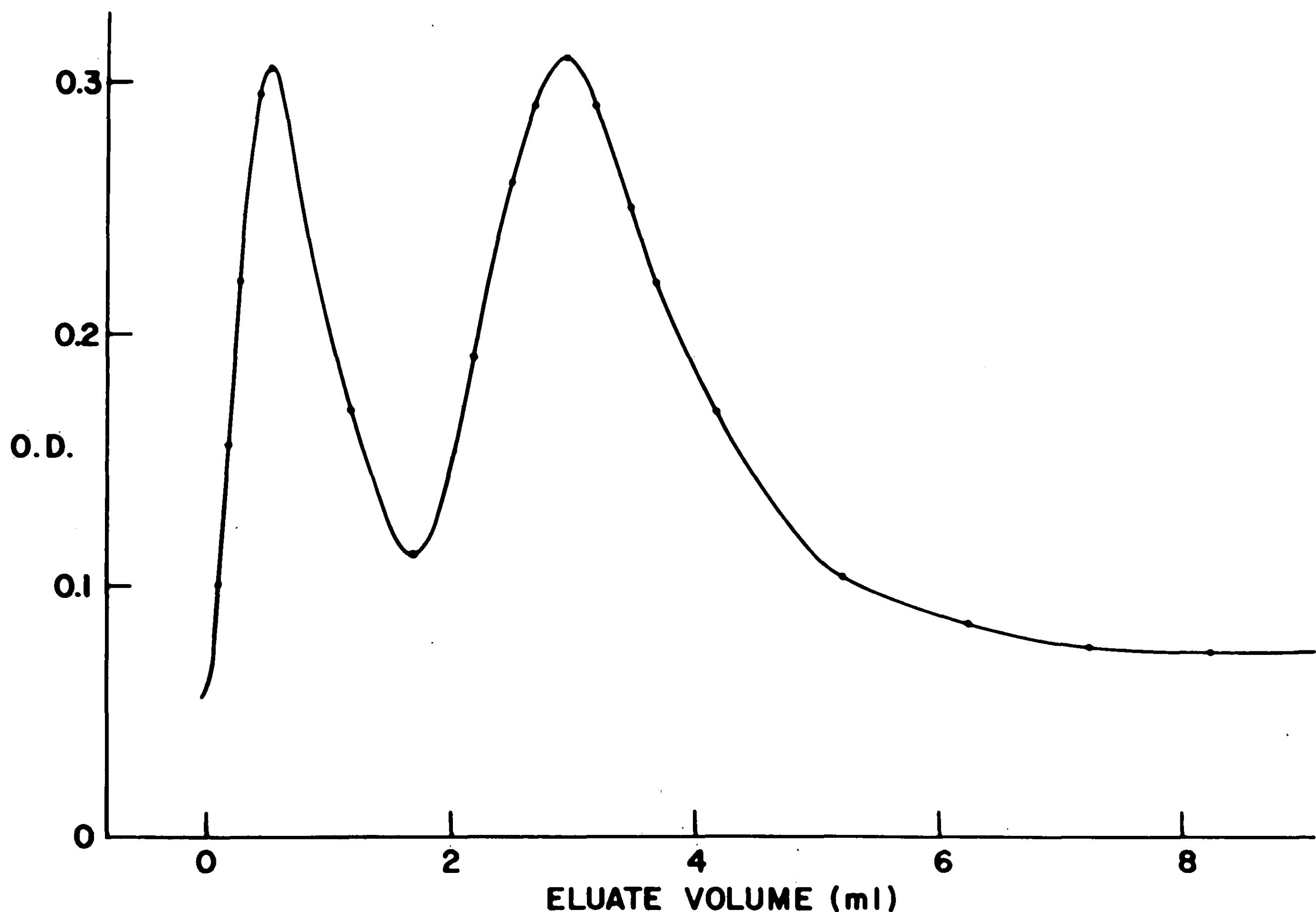
Molarity	Elution	0.05	0.05	0.06	0.065	0.05	0.06	0.07	0.083
	Resin	0.05	0.06	0.06	0.065	0.07	0.07	0.07	0.083
Resistance (Ω)	Elution	225	230	205	187	230	205	175	155
	Resin	235	205	205	187	175	175	175	155
Recovery A_{1c} (%)		6	<5	82	82	87	92	>100	>100
Pool Fast Hb (%)		2.4	<5	5.3	5.3	7.1	7.7	>15	>15
Observations			A_{1c} not eluted in 5 ml				visible bands		merged bands A_0 breakthrough

Resin was initially equilibrated to 0.05M buffer.

within the narrow range of 0.05M to 0.08M are shown in Table 1. Also in these experiments, we varied the ionic strength used to equilibrate the resin from 0.05M to 0.07M. The resistance of each buffer (at 19–21°C, see below) was recorded to provide an indication of ionic strength independent of weighing errors in the buffer preparation. The results shown in Table 1 indicate that a combination of equilibration and elution buffer molarities was found that would provide full recovery of the A_{1c} peak with separate bands of A_{1a+b}, A_{1c}, A_0, and A_2 visually apparent during elution and no contamination of the fast fraction with A_0. The elution profile using these optimal conditions is shown in Figure 3. This system, composed of resin equilibrated to 0.07M buffer and to 0.06M elution buffer (both pH 6.70–6.72 containing 0.01M KCN), was used for all subsequent studies.

Figure 4 shows the effect of increasing the elution volume on the percent recovery of purified A_{1c} and on the hemoglobin appearing in the glycohemoglobin fraction from the normal pool. In the system described above, HbA_{1a} began to elute after approximately 1.5 to 2.1 ml, followed by A_{1b}. The figure shows that, by 4 ml, recovery of A_{1c} was virtually complete.

FIGURE 3. Elution pattern of 416-nm absorbance as a function of elution volume using a nondiabetic hemolysate. The first peak was HbA_{1a+b}; the second peak was HbA_{1c}.



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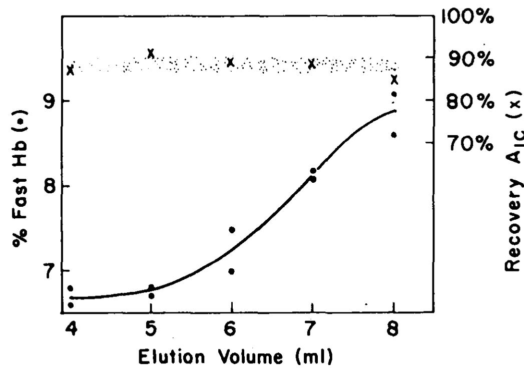

FIGURE 4. Effect of increasing elution volumes on the percent hemoglobin recovered in the glycohemoglobin fraction using 20 μl of a nondiabetic hemolysate (different patient than the one used in Figures 1 and 2) and on the recovery of purified A_{1c}. Other details are are described in the text.

A highly critical factor in the behavior of the small columns was temperature, as shown in Figure 5. At 7°C, purified A_{1c} remained at the top of the column and was not recovered at all in the fast fraction (5 ml). Conversely, at temperatures above 25°, substantial A-breakthrough was observed. Maximum recovery of A_{1c} without A_0 contamination required temperatures be kept between 19° and 21°C.

Several versions of quantitating percent glycohemoglobin after chromatography have been described.[3] In some methods, a second fraction (containing A_0 and possibly A_2) is eluted by a high phosphate, low pH buffer (0.25M, pH 6.4). Results can be calculated using the absorbances at 416 or 540 nm (see below) of the first and second fractions or of the first fraction and the total hemolysate (without chromatography): the glycohemoglobin absorbance is divided

FIGURE 5. Effect of temperature on the elution of hemoglobin into the glycohemoglobin fraction from a diabetic sample and on the recovery of purified A_{1c}. Other method parameters are described in the text.

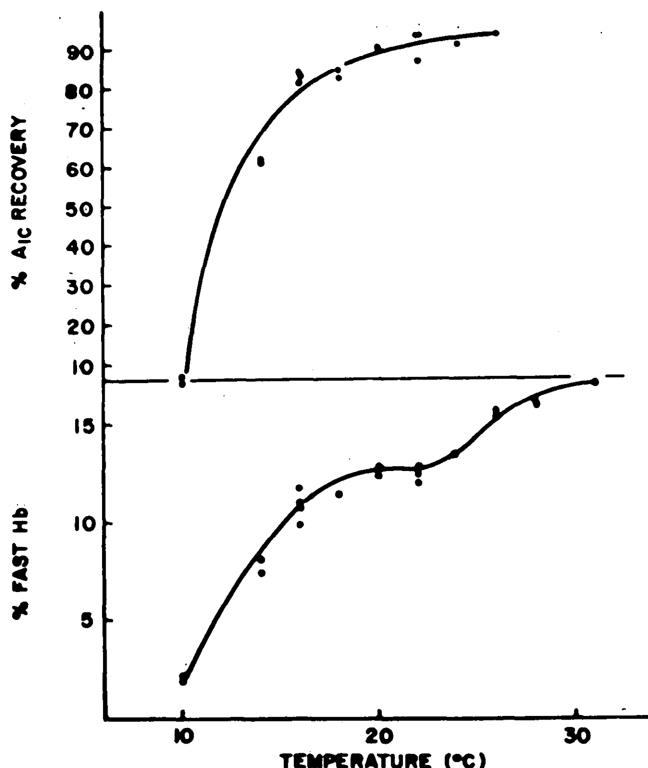

TABLE 2
Relative precision of absorbance measurements and calculations of percent glycohemoglobin (N = 5)

Wavelength (nm)	Calculation	Mean result (%)	Preliminary CV (%) (inter-run)
416	F/A	8.1	8.1
	F/T	7.2	10.2
540	F/A	7.4	18.9
	F/T	6.6	17.2

either by the sum of the absorbances of the first and second fractions (F/A) or by the absorbance of the total hemolysate (F/T). Table 2 is a list of the mean pool values and the precision using these calculations, the absorbances being determined on a Beckman 25 spectrophotometer. Precision of the 416-nm measurements was greater than that of the 540-nm measurements on the same sample. The F/T was less than the F/A ratio, because the total hemolysate included the A_2 fraction, which may not elute fully in the second fraction. These precision estimates differ from the final method precision described below, because the elution temperature was not controlled in this experiment; the data in Table 2 should be considered a comparative study. We found that the absorbance of the diluted hemolysate should be determined within one hour, because a rapid loss of absorbance occurs at high dilution.

The linearity of the method was determined by adding increasing amounts of purified A_{1c} to a normal hemolysate. Figure 6 demonstrates that linearity in precent glycohemoglobin extends from 7.6% to at least 18.2%. Recovery of the A_{1c} spiked to pools was about 90–95%.

The precision of the method was evaluated using prepared hemolysates stored at −70°C for up to 90 days. In independent runs over 10 days, the within-run coefficient of variation (C.V.) was 2.2% and the between-run C.V. was 4.8% using a nondiabetic adult pool (mean = 7.5% glycohemoglobin). Similarly, in a diabetic hemolysate (mean = 12.3% glycohemoglobin), within-run C.V. was 3.4% and the between-run C.V. was 5.1%.

Interference studies. Red cells from heparinized cord blood (containing high HbF) were centrifuged, washed three times with saline, and lysed by adding 1 vol of water and 2 vol of carbon tetrachloride; 20 μl of this hemolysate was added to the columns and eluted as described in ROUTINE METHOD. Almost *all* cord blood hemoglobin appeared in the glycohemoglobin fraction (80–94%). A normal pool containing 8% glycohemoglobin was diluted 1 + 1 with the cord blood hemolysate. Elution of the spiked pool showed one abnormal band eluting before HbA_{1a} and HbA_{1b} and another before HbA_{1c}; the glycohemoglobin fraction contained 30% of the total hemoglobin. Therefore, HbF appeared to be measured with the glycohemoglobin fraction, resulting in an elevation in the calculated percentage of HbA_1.

A human hemolysate containing HbC and HbS was prepared as described in METHODS, and 3.3% of the hemoglobin eluted in the fast fraction. This hemolysate was mixed (1 + 1) with a normal pool (7.9% glycohemoglobin). The fast fraction contained 6% of the hemoglobin in the mixture. This compared well with a theoretic yield of 5.6% in the gly-

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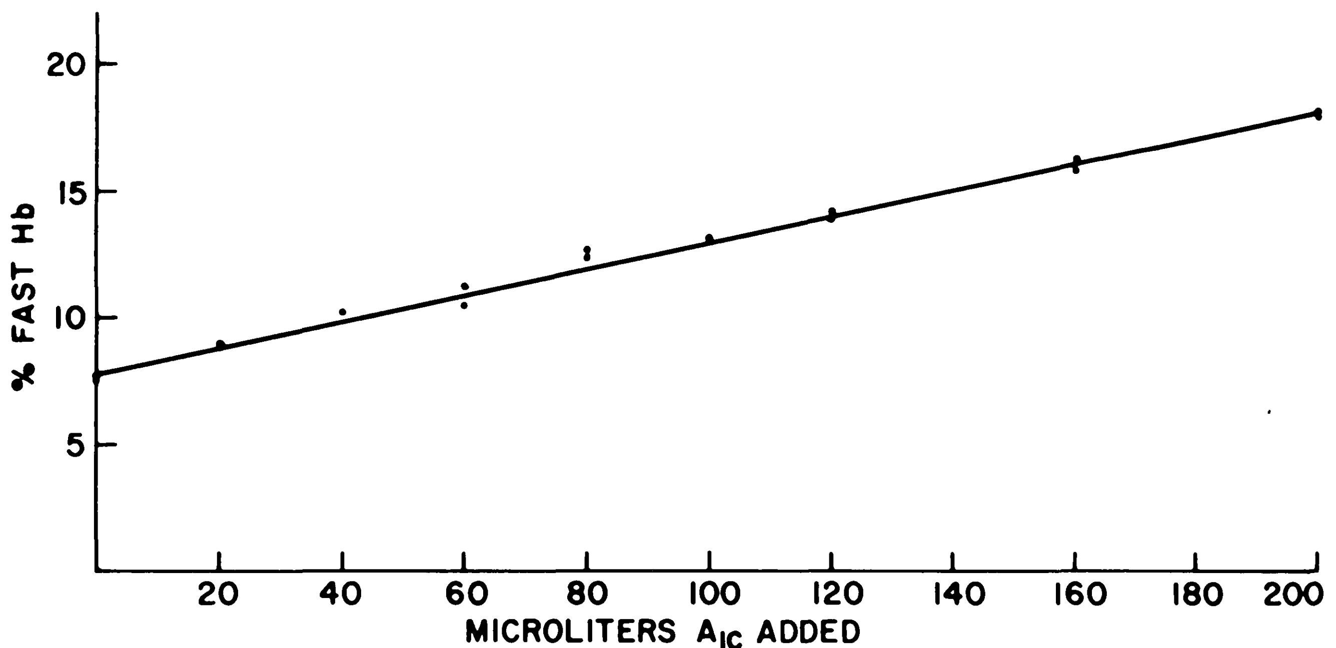
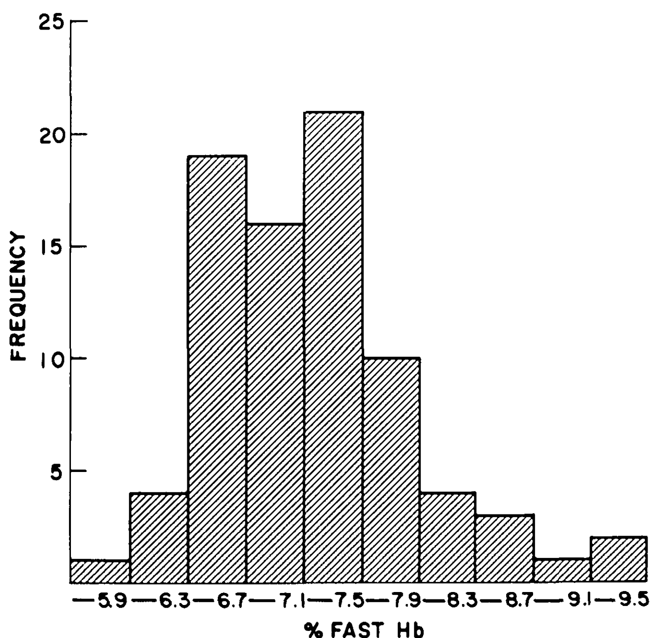


FIGURE 6. Linearity in percent glycohemoglobin with increasing amounts of purified A_{1c} added to a nondiabetic hemolysate. Elution conditions are described in the text.

cohemoglobin fraction, consistent with no interference. The abnormal hemoglobins remained at the top of the column with the A_0 and did not interfere in the fast fraction. Because the method did not depend on complete elution of a second fraction from the column, as required in other procedures,[3] no methodologic interference was observed by HbC and HbS.

The effect of high bilirubin in blood on glycohemoglobin was tested by adding 3.4 μg of bilirubin from a blood sample containing high bilirubin to the column; it eluted entirely in the glycohemoglobin fraction. The glycohemoglobin result increased from 9.5% to 10.8% in the normal pool when 20 μl of a high bilirubin serum was added to the hemolysate.

FIGURE 7. Distribution of the glycohemoglobin results in nondiabetic adults: 95% confidence limits indicate a range of 6.08–8.8% (mean, 7.2%) glycohemoglobin.

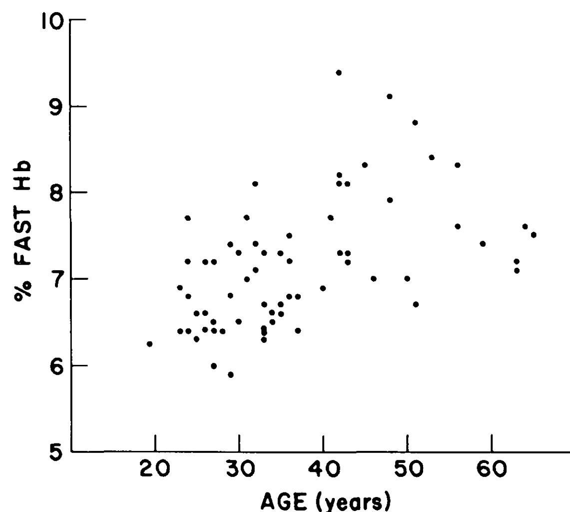


Reference range. The distribution of the glycohemoglobin results from 85 nondiabetic adults is shown in Figure 7. The data fit an arc sinh distribution with a non-Gaussian reference range estimate[15] of 6.0–8.8%. A trend toward increasing glycohemoglobin with increasing age is shown in Figure 8.

Clinical study. The results of a small study of treated diabetics attending an outpatient diabetes clinic are shown in Figure 9. A few patients in the group, rated as well controlled, exhibited glycohemoglobin results within the normal range. No overlap to the normal range was found in the less well-controlled groups of patients.

Sample stability. Other glycohemoglobin methods reported to date require fresh specimens or refrigerated storage (4°C). Since this storage condition is not convenient

FIGURE 8. Percent glycohemoglobin as a function of age in nondiabetic subjects. Correlation coefficient = 0.46.



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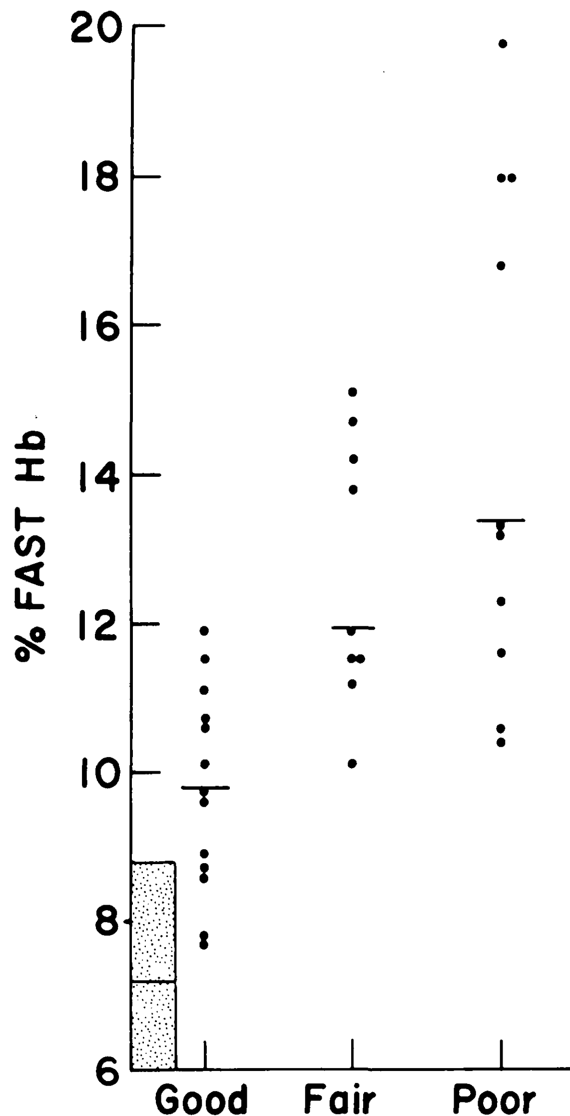

FIGURE 9. Distribution of glycohemoglobin results from 32 diabetics rated to be in good, fair, and poor control compared with the nondiabetic range. Group medians are indicated by the horizontal lines.

for monitoring diabetic outpatients, other stable conditions were sought (Table 3). These preliminary results suggested that whole blood samples stored at 30°C, −20°C, or −70°C would show elevated results. The red cell hemolysates shown in Table 3 appeared to be stable for at least 10 days stored at 4° or −70°C. Subsequently hemolysate pools stored at −70°C were used to monitor the precision of the assay (see above) and did not show any signs of deterioration for at least 3 mo.

In contrast with results from stored whole blood, storing the separated *red cells* frozen at −20°C (Figure 10) for 6 days did not affect glycohemoglobin results in samples drawn from normal and diabetic adults (N = 31). None of the results of the 31 samples tested was significantly affected by frozen storage of the red cells before hemolysate preparation.

DISCUSSION

The chromatographic separation of the glycohemoglobin fraction (A_{1a}, A_{1b}, and A_{1c}) from the main hemoglobin peak

TABLE 3
Storage effects on glycohemoglobin in a single sample and recovery of purified HbA_{1c}

Anti-coagulant	Sample	% Glycohemoglobin				
			After storage* at			
		Day 0	30°C	4°C	−20°C	−70°C
Heparin	Whole blood	6.8	13.5	8.5	11.0	8.9
	Hemolysate		16.5	7.7	11.6	7.2
EDTA	Whole blood	7.0	18.5	8.0	10.6	9.0
	Hemolysate		29.3	7.5	11.9	7.4
Oxalate	Whole blood	7.5	11.8	7.8	10.5	8.7
	Hemolysate		16.5	7.6	13.3	7.8
		Percent recovery				
	Purified A_{1c}	94	67	93	95	94

* A_{1c} for 5 days, whole blood for 7 days, hemolysate for 10 days.

(A_0) was exquisitely sensitive to several variables: pH, ionic strength, temperature, and elution volume, as suggested by recent communications.[16] These variables must be rigorously and consistently controlled, i.e., the pH to ±0.02 units, and an operating temperature range of 19–21°C must be maintained. It is recommended that the resistance of all buffers (at controlled temperatures) be used to monitor and adjust ionic strength within the limits defined in METHODS. Even within the tolerances defined, the effects of these variables are observed as trends in the daily quality control pool results. Workers who have not controlled these variables report difficulties in achieving reproducible results.[6,16,17]

The good separation of A_{1a+b} from A_{1c} shown in Figure 3 represents a significant improvement over the single merged A_1 peak previously thought to be characteristic of small column methods.[16] The separation characteristics of the columns can be observed visually during elution and are fundamental to the accuracy and precision of glycohemoglobin determinations because the potential interference by HbA_0 is not apparent in the absorbance characteristics of the fractions.

During development of the method, we discovered that intact cells elute in the fast fraction and the resulting turbidity caused the percentage of glycohemoglobin to be biased high by as much as twofold. The effect of this turbidity on the absorbance could be removed by centrifugation of the eluates or by the more complete hemolysis obtained in the present procedure.

HbF was nearly completely recovered in the fast fraction. Glycohemoglobin determinations, therefore, may not be useful in monitoring pregnant patients, since HbF may be produced in varying amounts throughout pregnancy. This finding has also been reported in other chromatographic methods in which correction for HbF was applied.[8] This procedure is hampered by the poor precision of HbF measurements.[20] While no methodologic interference was apparent in the hemolysate containing HbC and HbS, the glycohemoglobin was low (well below the normal range); this may mean that HbS and/or HbC were not glycosylated at the same rate as A_0, or that the glycosylated S or C did not elute in the glycohemoglobin fraction, or that red cell survival was reduced in the patient.[18] Therefore, there may be a physio-

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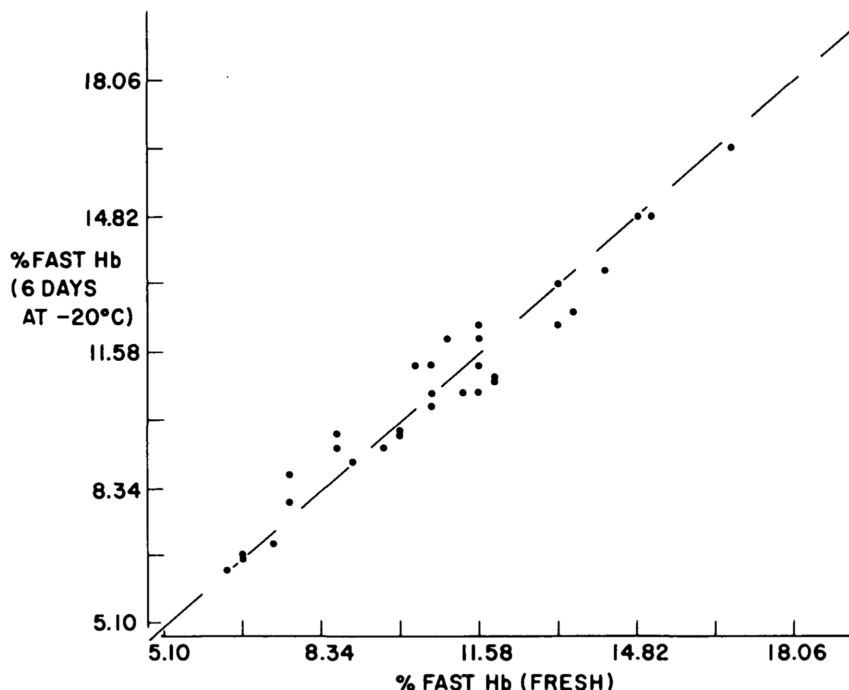

FIGURE 10. A comparison of glycohemoglobin results obtained from hemolysates of fresh red cells and of red cells frozen for 6 days at −20°C using diabetic and normal samples. A slope of 1.0 is shown by the dashed line.

logic (*in vivo*) interference from HbC and HbS, leading to difficulties in interpreting such results. However, results from such patients could be compared with previous determinations for long term clinical monitoring.

In accord with other reports,[19] a trend was found in which glycohemoglobin increased with age in nondiabetics. This factor may partly explain the differences in the normal ranges reported and in the degree of overlap of diabetic and nondiabetic results[15,19] (and see below).

The glycohemoglobin found in 34 treated diabetic patients grouped according to hyperglycemic control shows that values from only four patients believed to be in good control fell within the nondiabetic range. None of the patients rated fair or poor overlapped the reference range, and group medians reflected control ratings. The discrimination among groups was somewhat better than in the previous reports[15,19] and may be a result of our control of methodologic variables. Substantial overlap occurred among the diabetic groups and probably reflected the acknowledged difficulty in accurately assessing the status of many diabetics on the basis of clinical judgment and conventional laboratory testing.[2]

Sample stability is a potentially serious problem in routine patient monitoring that has received little attention in other reports. The statistical significance of published results has not been presented, even for the most commonly suggested storage condition of whole blood at 4°C. Based on the preliminary studies described, storage of whole blood for seven days did not appear promising at any temperature, with the possible exception of 4°C. The elevation in the glycohemoglobin fraction observed after storage of whole blood or hemolysates at 30°C could be attributed to hemoglobin breakdown products that co-elute with A_{1a+b}. These contaminants may begin to appear after as early as a few hours at room temperature.[7] This artifactual elevation in glycohemoglobin would interfere with the interpretation of samples from outpatients or other patients not in close proximity to the laboratory, because there is no way a priori to distinguish between a real elevation in the fast fraction and an artifactual elevation secondary to improper sample storage.

The demonstration that red cells could be stored at −20°C for at least six days before analysis (Figure 10) allowed convenient storage, especially for samples likely to encounter some delay in reaching the laboratory. As shown in Table 3, the stability of frozen whole blood was highly questionable and was not pursued. We have observed that hemolysates may be stored at −70°C for several months without apparent deterioration and are useful as quality control pools. The prepared A_{1c} standard was dialyzed against the cyanide-containing eluting buffer after chromatographic purification, and it was relatively stable in this form, except at 30°C.

In summary, the variables affecting the accuracy and precision of the chromatography and the quantitation of glycohemoglobin were examined in an effort to avoid methodologic artifacts so that the proper role of this laboratory test in the management of diabetic patients could be evaluated. The method described has been optimized with respect to those variables and has been fully characterized by linearity, recovey, precision, and interference studies. Particular attention has been directed toward stability questions, and the method was successfully applied to a small group of diabetic patients.

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