Comparison of Percent Total GHb With Percent HbA_{1c} in People With and Without Known Diabetes

FRANK Q. NUTTALL, MD, PHD

OBJECTIVE — To directly compare results obtained using an ion-exchange high-performance liquid chromatography (HPLC) HbA_{1c} method used in the Diabetes Control and Complications Trial with two different affinity chromatography methods in which "total GHb" is determined.

RESEARCH DESIGN AND METHODS — Blood was obtained from a large number of people with and without known diabetes. The specimens were divided and assayed for HbA_{1c} and for total GHb. Total GHb was determined using a semi-automated gravity-elution boronate affinity chromatography method and an automated boronate affinity HPLC method. The results obtained with the two methods were also compared.

RESULTS — In subjects without known diabetes, the mean percentage HbA_{1c} and the range of values were similar to the total GHb values in the same subjects when assayed using the semi-automated affinity gravity-elution method (mean 5.2 ± 0.4 and 5.1 ± 0.4% [SD], respectively). With the affinity HPLC method, results were 5.3 ± 0.4%. The similarity in results was surprising. However, analysis of the data suggests that a large proportion of the material in the HbA_{1c} fraction measured using this ion-exchange HPLC method is not GHb, as pointed out by others. Although the results were similar in people without known diabetes, in the people with diabetes, the incremental increase was ~25% greater for the total GHb when compared with the increase in HbA_{1c}. When corrected for the non-GHb being measured by the HbA_{1c} method, it can be calculated that ~40% more GHb is measured using affinity chromatography over the entire range of GHb values.

CONCLUSIONS — The similarity in the mean and range of percent HbA_{1c} and in percent total GHb using these different methods can be attributed to two factors: *1*) the HbA_{1c} ion-exchange method measures only ~50–60% of the total GHb present, and 2) ~40–50% of the material being measured in the HbA_{1c} fraction is not GHb, i.e., offsetting factors fortuitously resulted in values similar to the more specific affinity methods. The greater incremental increase in percent total GHb compared with percent HbA_{1c} in people with diabetes can be attributed to the greater amount of GHb being measured with the affinity methods.

Diabetes Care 21:1475–1480, 1998

Presently, there are over 30 different GHb methods used to estimate the plasma glucose concentration integrated over an extended period of time (1). We have had the opportunity to directly compare the results of two boronate affinity column chromatographic methods for measuring the percent total GHb with the percent HbA_{1c} determined by ion-exchange high-performance liquid chromatography (HPLC) in subjects without known diabetes and in subjects with diabetes. The HbA_{1c} determination was done in the laboratory that served as the central laboratory for the Diabetes Control and Complications Trial (DCCT). The two affinity methods were a gravity-elution method and an HPLC method done in our laboratory.

Ion-exchange HPLC depends only on a change in the charge of the globin proteins in hemoglobin, whereas boronate affinity columns specifically bind the coplanar vicinal hydroxyl and/or carbonyl groups of hexoses (sugars) attached to the globin proteins. That is, the affinity methods measure only the fraction of hemoglobin to which a sugar is attached. With the ion-exchange method, anything that changes the charge of the hemoglobin molecules is measured. This may or may not be a sugar. Thus, total GHb determined by affinity chromatography should be a better indicator of the mean blood glucose concentration to which erythrocytes are exposed over their lifetime in the circulation. Because the HbA_{1c} fraction represents only a portion of the total glycosylation of the globin protein, the percent total GHb should be considerably greater than the percent HbA_{1c}.

RESEARCH DESIGN AND METHODS

— Blood was obtained from employees, medical students, house staff, and hospital volunteers in a nonfasting state. All were in good health. Specifically, none had a history of a hematological disorder, liver disease, thyroid disease, or cardiovascular disease. Subjects with diabetes were attending diabetes or general medicine clinics and were not selected. To determine a reference interval for the gravity-elution boronate affinity method, blood samples were obtained from 298 subjects. Ages were available for 216 subjects. The age range was 19–86 with a mean of 45 years; 52% were women, and 48% were men. Subsequently, a reference interval for the automated affinity HPLC system was established using blood samples obtained from 148 subjects. The age range was 17–79 with a mean of 45 years; 57% were women, and 43% were men. The reference interval for

From the Metabolic Research Laboratory and the Section of Endocrinology, Metabolism and Nutrition, Minneapolis Veterans Affairs Medical Center; and the Department of Medicine, University of Minnesota, Minneapolis, Minnesota.

Address correspondence and reprint requests to Frank Q. Nuttall, MD, PhD, Section of Endocrinology, Metabolism and Nutrition (111G), Minneapolis VA Medical Center, One Veterans Dr., Minneapolis, MN 55417.

Received for publication 8 December 1997 and accepted in revised form 14 May 1998.

Abbreviations: CV, coefficient of variation; DCCT, Diabetes Control and Complications Trial; HPLC, high-performance liquid chromatography.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

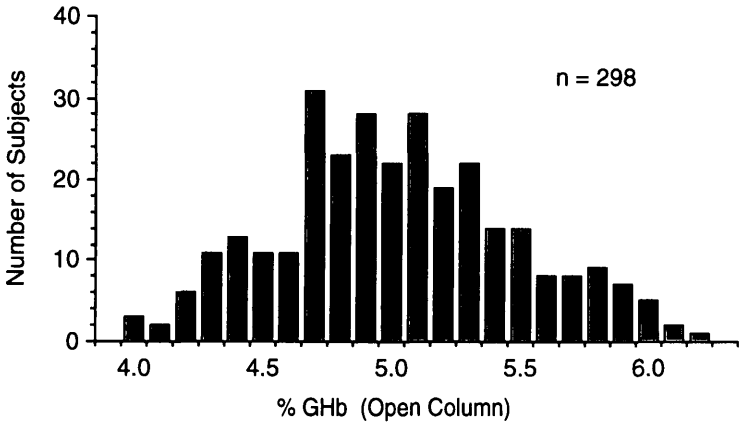

Figure 1—*Distribution of data from subjects without known diabetes using a gravity-elution boronate affinity column method for determining percent total GHb.*

the ion-exchange HPLC (HbA_{1c}) method had been previously determined by the laboratory service at the University of Minnesota Medical Center using data obtained from 164 subjects without known diabetes. The age range was 13–39 years.

For comparison of the results obtained with the ion-exchange HPLC (HbA_{1c}) method with those of the gravity-elution boronate affinity column method, blood samples were obtained in 54 subjects without known diabetes and 98 diabetic subjects.

For comparison of the results obtained with the ion-exchange HPLC (HbA_{1c}) method with those of the automated boronate affinity HPLC method, the same blood samples were used; however, the number of diabetic subjects studied was increased to 114. The age range of the 54 subjects without known diabetes was 19–86 with a mean of 48 years. Blood samples were divided. One part was processed and analyzed for HbA_{1c} by the laboratory service at the University of Minnesota Medical Center using a Bio-Rex 70 ion-exchange resin HPLC method. Another part was processed and analyzed for total GHb using a gravity-elution boronate affinity column method using columns supplied by Isolabs (Akron, OH), and/or it was analyzed by an automated HPLC boronate affinity column method supplied by Primus (Kansas City, MO). The processing and analysis of samples was as directed by the suppliers.

The gravity-elution boronate column chromatography method is a semi-automated or manual method. It is relatively time-consuming, and the turnaround time is long. Minor variations in column efficiency in separating the GHb fraction from the remaining hemoglobin have been reported (2). Recently, automated boronate affinity HPLC methods have become available. HPLC has the advantage of greater speed (assay time 2 min), small sample size (8 µl blood), and excellent reproducibility.

Blood samples were collected in tubes containing dipotassium EDTA as an anticoagulant. Blood samples were stored in a refrigerator at 4°C. Generally, the analyses were done within 3 days of obtaining the samples and always within 7 days. In those analyses in which stability was studied, the aliquoted samples were kept in a refrigerator at 4°C for up to 54 days. In the longer-term stability study, samples were aliquoted, diluted with the diluent used in the assay, and kept at −70°C.

Data are presented as a percentage of total hemoglobin. Graphing was done using the Cricket Graph, version 1.3 software (Malvern, PA) for the Macintosh computer.

For all samples from people without known diabetes, the distribution of data obtained using each method was shown to be compatible with a Gaussian distribution. Skewness and kurtosis were not statistically significant. Therefore, for comparative purposes, the mean and 2 SDs from the mean have been used in comparing data sets. This range has been defined as the reference interval.

RESULTS — Distribution of the percent total GHb results obtained in the 298 subjects without known diabetes using the gravity-elution boronate column chromatography method is presented in Fig. 1. The median was 5.1%. The mean was 5.0% with an SD of 0.46%. A reference interval based on 2 SDs from the mean is 4.1–5.9%.

In the 148 subjects without known diabetes, the determination of the percent total hemoglobin results obtained using the automated affinity HPLC system was 5.6 with an SD of 0.44%. A reference interval based on 2 SDs is 4.7–6.5% (Fig. 2). Thus, the results were 8% higher than with the gravity-elution affinity method. This difference was statistically significant ($P < 0.001$). The reason for the difference is not known. It may be due to a more complete elution of the GHb fraction with the affinity HPLC method. The mean percentage HbA_{1c} previously determined at the University of Minnesota Medical Center was 5.00 with an SD of 0.36%. A reference interval based on 2 SDs from the mean is 4.3–5.7%. Thus, the means and range of values obtained with the gravity-elution affinity method and the HbA_{1c} method were similar. The results were only ~8% higher with the affinity HPLC method, even though the two affinity methods measure total GHb and not just the HbA_{1c} fraction.

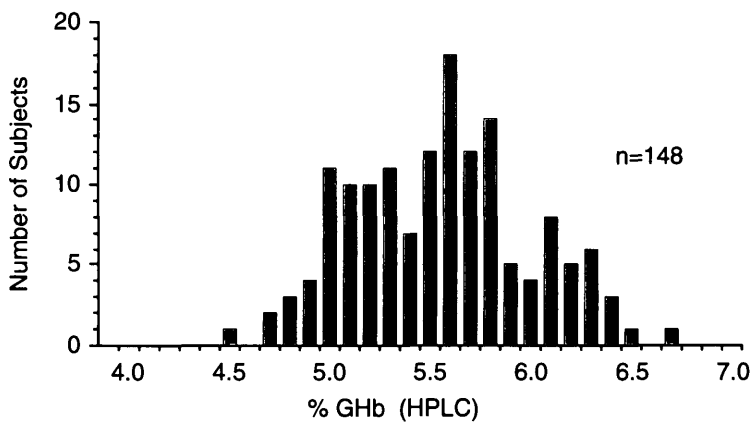

Figure 2—*Distribution of data from subjects without known diabetes using a boronate affinity HPLC method for determining total GHb.*

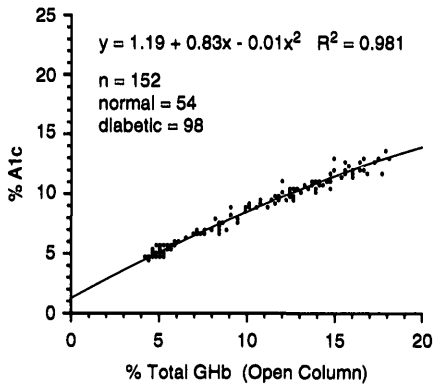

Figure 3—*Correlation of results in subjects with and without known diabetes using an ion-exchange HPLC (HbA_{1c}) method and a gravity-elution boronate affinity column total GHb method.*

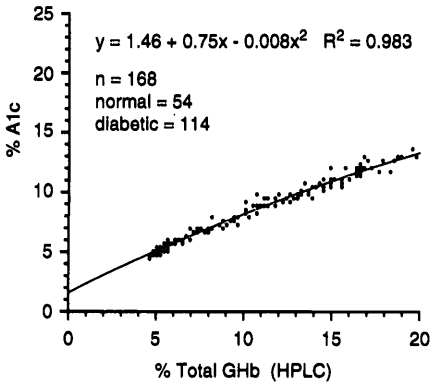

Figure 4—*Correlation of results in subjects with and without known diabetes using an ion-exchange HPLC (HbA_{1c}) method and a boronate affinity HPLC total GHb method.*

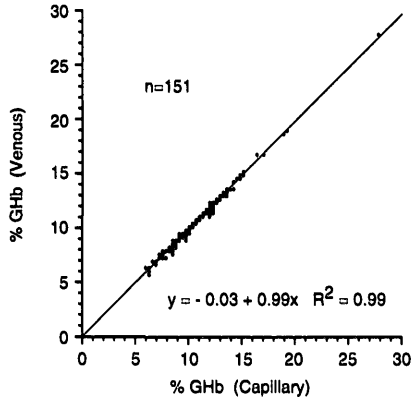

Figure 5—*Correlation of results using a boronate affinity HPLC total GHb method and venous versus capillary blood.*

Because the results were not as anticipated, we directly compared the total GHb results obtained by the two affinity chromatography methods with the HbA_{1c} results obtained at the University of Minnesota Medical Center using divided blood specimens. Samples from both subjects without known diabetes and diabetic subjects were included.

In the 54 subjects without known diabetes, the mean percentage of total GHb determined by the gravity-elution affinity method was 5.1 with an SD of 0.4%. The mean percentage of total GHb determined by affinity HPLC was 5.3 with an SD of 0.4%. The mean HbA_{1c} in the same subjects was 5.2 with an SD of 0.4%. The respective medians were essentially identical to the means.

Thus, the means and the reference intervals using these entirely different methods were again very similar when blood samples from the same subjects were analyzed. The difference between the results obtained with the affinity HPLC method and the other two methods, though small, was statistically significant ($P < 0.008$).

When the data obtained in subjects without known diabetes using the gravity-elution affinity and the HbA_{1c} were combined with the data in subjects with diabetes and plotted, there was a slightly curvilinear relationship between the percent total GHb determined by the gravity-elution affinity method and the percent HbA_{1c} results (Fig. 3), and a significant amount of nonspecific material was present with the HbA_{1c} method. The slope of the line indicated a greater incremental increase in total GHb compared with the increase in HbA_{1c} in the subjects with diabetes.

The same 152 samples were assayed using the automated affinity HPLC method and compared with the gravity-elution affinity column method. The intercept was essentially zero. In addition, there was a linear relationship between the results of the two methods in contrast to the curvilinear relation between the ion-exchange HbA_{1c} and the gravity-elution affinity total GHb data. However, the slope was 1.08, indicating that the values were slightly higher using the boronate HPLC method (data not shown).

The affinity HPLC total GHb results were compared with the HbA_{1c} ion-exchange HPLC results using the same divided blood samples to which data from 16 additional subjects with diabetes were added (Fig. 4). The equation relating the two was again curvilinear and again indicated the presence of significant nonspecific material with the HbA_{1c} method. The slope of the line also indicated a greater incremental increase in total GHb compared with the HbA_{1c} results.

Because the affinity HPLC total GHb method uses such a small sample of blood, we compared results obtained using capillary blood (fingerstick) with venous blood obtained at the same time. The results were identical (Fig. 5). We have also found that blood specimens may be stored for up to 56 days at 4°C without affecting the results (data not shown).

Differences in performance of the columns used in analysis of HbA_{1c} and total GHb may affect the reproducibility of results over time. Therefore, we determined the GHb fraction in frozen blood samples analyzed repeatedly over a 17- to 25-month period using the affinity HPLC method. During this time period, the columns were changed 15 times. The results were highly reproducible. For a sample with a mean GHb of 5.5% determined monthly for 17 months, the SD was 0.10% and the coefficient of variation (CV) was 1.8%. For a sample with a mean of 10.1% determined monthly for 25 months, the SD was 0.18% and the CV was 1.8%. For a sample with a mean of 16.9% determined 21 times, the SD was 0.18% with a CV of 1.1%.

CONCLUSIONS — The amount of glucose derivatized to the globin molecules of hemoglobin has become a widely used means of estimating the circulating glucose concentration integrated over a period of several weeks in people with diabetes mellitus. Usually, it is reported as a percentage of the total hemoglobin present.

At the present time, determination of HbA_{1c} by ion-exchange HPLC represents the most commonly recommended method for determining the fraction of globin molecules to which a glucose molecule is permanently attached through conversion to a ketoamine. In fact, it has been referred to as the "gold standard" (3). It is also the method used in the DCCT. In addition, it is the method against which the results for all other methods are being standardized in the U.S. (4). Nevertheless, determination of the entire sum of glycosylated species theoretically should be a better representation of the integrated glucose concentration. The data we have obtained, particularly with the automated affinity HPLC method, indicate that this is indeed the case.

The globin protein in hemoglobin consists of two α-chains and two β-chains.

Glucose may bind to the NH_2-terminus of the chains or to lysine amino acids within the chains. After undergoing an Amadori rearrangement, both result in a ketoamine adduct. Thus, glucose derivatization occurs at several sites. A major site of glycosylation is the NH_2-terminus of the β-chains, and it is this glycosylated species that appears in the HbA_{1c} fraction when hemoglobin molecules are analyzed by ion-exchange chromatography. Irreversible glycation at these sites is now the definition of HbA_{1c} used by the International Federation of Clinical Chemists working group on HbA_{1c} standardization (5). It has been estimated (6,7) that this species makes up about 50% of the glucose derivatized irreversibly to the globin chains of hemoglobin.

However, ion-exchange chromatography, which is used for determining the HbA_{1c} fraction, is a nonspecific method. It depends only on changes in net charge of the protein and/or changes in protein conformation for separating subspecies. These changes may occur as a result of the attachment of any adduct that neutralizes the charge of an ionized amino acid or changes the conformation of the protein. In the case of the HbA_{1c} fraction, any adduct that attaches to the amino group of the NH_2-terminal amino acid of the β-chains of globin may be included in the HbA_{1c} fraction. Carbamylation of the globin protein in people with uremia, acetylation secondary to aspirin ingestion, and ascorbatylation due to vitamin C ingestion have all been identified and may be included in the HbA_{1c} fraction as measured using ion-exchange chromatography (7–11). Other unidentified adducts are likely to be present (12). With ion-exchange chromatography, it is also difficult to completely eliminate contamination with small amounts of fetal hemoglobin. In addition, 500 hemoglobin variants resulting from specific amino acid substitutions have been identified; 50 of these could potentially affect the HbA_{1c} fraction as determined using ion-exchange chromatography (13).

With the boronate affinity column methods currently in use, only the ketoamine product of the Amadori rearrangement of the attached glucose is measured, i.e., fructosylated globin. The site (sites) where glucose attached is not important, since it is the fructose attached to the protein and not the protein that binds to boronate. Thus, the data represent "total glycosylation" of the hemoglobin. Hemoglobin variants have not been reported to affect the results. Derivatization of globin by nonmonosaccharides could lower the total glycosylation of the hemoglobin, but in practice, this generally has not been a problem. Nevertheless, ingestion of 1.0 g ascorbic acid daily has been reported to lower the percent GHb in normal subjects (10).

Because the affinity method and the ion-exchange method measure different products, at least in part, we were surprised to find the range of values for each to be similar in people without known diabetes. The answer to this is now apparent. Flückiger (6) and others (11,14) obtained data indicating the presence of large amounts of nonglycated products being measured in the HbA_{1c} fraction of hemoglobin obtained from people without known diabetes. This has now been confirmed using a high-resolution ion-exchange method (15) and a mass-spectrometry method (5,12,16). Indeed, nearly half of the material being measured in the HbA_{1c} fraction does not represent glycosylation of the NH_2-termini of the β-chains. Thus, our observation that the reference intervals for HbA_{1c} and for total GHb are similar can be explained by two offsetting factors: *1*) only about 50% of the total GHb species are found in the HbA_{1c} fraction (17,18), and 2) ~50% of the material measured as HbA_{1c} by ion-exchange chromatography is not due to glycosylation of globin in the fraction. It is only fortuitous that these two factors were approximately equal and offsetting. It also explains why the data comparing the total GHb obtained using affinity chromatography and the HbA_{1c} data obtained by the Bio-Rex 70 ion-exchange HPLC method used in the DCCT indicated the presence of significant nonspecific substances being measured with the HbA_{1c} method (Figs. 3 and 4).

The regression line between the two methods (Figs. 3 and 4) was also significantly different from 1, indicating that the affinity method distinguishes greater differences in the amount of fructosylation of the globin, particularly in the range generally present in people with diabetes, as noted by others (19–22). Because the relationship between the HbA_{1c} fraction and total GHb was curvilinear, it may possibly be due to glucose becoming attached to lower-affinity lysine sites at higher glucose concentrations, i.e., there may be an ordering of affinities for binding as reported previously (23). Alternatively, it may merely indicate a linear increase in glycation at the NH_2-terminus of the β-chains superimposed on a constant amount of nonspecific material being measured.

In support of the latter concept is a recent report in which the relationship between the percent HbA_{1c} and the mean blood glucose profiles obtained during the 9 years of the DCCT was determined over the entire range of values. The equation that best fits the data was one in which 60.6 mg/dl was subtracted from the product of 31 mg/dl per percent HbA_{1c} and the actual percent HbA_{1c} measured to determine the mean blood glucose value (24).

This suggests that for all of the percent HbA_{1c} values obtained in the DCCT using the Bio-Rex 70 ion-exchange HPLC method, ~2.0% HbA_{1c} should be subtracted to obtain a true correlation of the HbA_{1c} results with the integrated glucose value reported in the DCCT. This is very close to the 40–50% of nonspecific material reported to be present in the percent HbA_{1c} in people without known diabetes using this method.

However, the relationship may be more complex. Recently, the percent HbA_{1c} represented by glycation of the NH_2-terminal ends of the β-chains has been determined using a highly specific and reproducible mass spectrometry method coupled with isolation of the six amino acid NH_2-terminal ends of the β-chains of globin. When the results were correlated with the results obtained using a mono-S ion-exchange chromatographic method, which is more specific than the ion-exchange chromatographic method used in the DCCT, the actual percent HbA_{1c} was overestimated at low percent HbA_{1c} values but underestimated at high values (5).

In any regard, the differences in values for percent total GHb and for percent HbA_{1c} in people with diabetes in the present study were clinically significant. The present data indicate that a 7.2% HbA_{1c} value, which was the mean for the intensively treated group in the DCCT (25), would be ~8.5% with the HPLC affinity method, even though the reference interval was only modestly higher with the latter method (Fig. 2). A value of 9.0%, which was the mean HbA_{1c} value in the conventionally treated subjects in the DCCT, corresponds to ~11.5% with the HPLC affinity method. An HbA_{1c} value of 10% would correspond to 13.5%.

The CV for the affinity HPLC total GHb method is equal to or superior to that for the ion-exchange HbA_{1c} method (26), even though the range of values deter-

mined with the affinity HPLC method is expanded. Thus, differences in mean blood glucose values integrated over several weeks to months may be determined with greater precision with the latter method.

It has been calculated, based on DCCT data, that for each 1% increase in HbA_{1c} above the normal range, there is an increase of ~30–31 mg/dl in integrated blood glucose concentration (provided 60.6 mg/dl is subtracted from the integrated glucose concentration) (3,4,24,27). Based on the correlation between the HbA_{1c} and total GHb data obtained using the affinity HPLC method, a 1% increase in total GHb would then represent an increase of only ~17–18 mg/dl (grossly 1% = ~1 mmol/l glucose change). A correction factor is not necessary, and preliminary data indicate that this relationship is present over the entire range of GHb values. However, all of these calculations should be considered estimates only, since the variance for the GHb in relationship to the mean blood glucose values is rather large (24,28).

Current attempts to standardize GHb testing and/or the development of algorithms that would allow interconversion of results using one method with another should be strongly endorsed (5,29), although the practicality of attempting to adjust results from one method to that of another has been questioned (30,31). It also has been suggested that the values obtained using various GHb methods be converted to the HbA_{1c} values obtained using the ion-exchange HPLC method used in the DCCT (29,32). Calibrating standards have been developed for several levels of HbA_{1c}. I would like to propose that the total GHb data obtained using the affinity HPLC not be converted to HbA_{1c} values obtained using this method. As indicated above, there are several inherent deficiencies with the ion-exchange HPLC determined HbA_{1c}, both practically and conceptually. In addition, sensitivity in differentiating different integrated glucose concentrations is likely to be less.

In a recent editorial (33), it was indicated that methodologies for determining GHb have progressed to the point where a decision needs to be made. Should we continue to use the ion-exchange HbA_{1c} method used in the DCCT as a "designated comparison method" for determining percent GHb, even though there are serious limitations with the method, and then attempt to reference all methods to it, or should we move to more specific methods with reference intervals that are different and then assume clinicians will relearn the clinical reference intervals and clinical decision points?

In our own institution, the latter option has been adopted and has not been a problem. After review of the analytical systems commercially available, the automated boronate affinity HPLC system was determined to provide data that best meet the needs of our clinicians. The reproducibility of the method is equal to, or superior to, the ion-exchange HPLC (HbA_{1c}) methods. Stable results are obtained over an extended period of time, long-lasting controls are available, calibration with an independent ion-exchange method is not necessary, specificity is excellent, and the results in relation to blood glucose control and the DCCT results are easily derived. If highly specific methods for determining the glycation of specific sites on the globin molecule become widely available, then this decision will need to be reassessed.

Acknowledgments— This study was supported by Merit Review Research Funds from the Department of Veterans Affairs.

The author would like to thank Dr. Mary C. Gannon for assistance in data analysis and for critically reviewing the manuscript; Drs. Michael Steffes and John Eckfeldt from the University of Minnesota Medical Center Pathology Department for providing the HbA_{1c} data and for useful discussions; the volunteers and Mary Adams, MT, for making this study possible; and Claudia Durand for expert secretarial assistance.

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