Effects of Sickle Cell Trait and Hemoglobin C Trait on Determinations of HbA_{1c} by an Immunoassay Method

WILLIAM L. ROBERTS, MD, PHD
MARY MCCRAW, MT(ASCP)
CURTISS B. COOK, MD

OBJECTIVE — A number of studies, including the Diabetes Control and Complications Trial (DCCT), have shown that good glycemic control, as assessed by GHb measurements, can reduce the chronic complications of diabetes. The National Glycohemoglobin Standardization Program (NGSP) was established to insure that GHb measurements by different methods were comparable and could be related to the candidate reference method used in the DCCT. The measurement of HbA_{1c} in patients with Hb variants is one area not directly addressed by the NGSP. Therefore, we assessed the comparability of two DCCT-traceable methods in samples with Hb variants.

RESEARCH DESIGN AND METHODS — Samples containing HbAA, HbAC, and HbAS were collected from diabetic and nondiabetic patients. HbA_{1c} concentrations were measured by a high-performance liquid chromatography method (Bio-Rad Diamat) and an immunoassay that is suitable for use in a physician's office (Bayer DCA 2000).

RESULTS — The two methods compared well for samples with HbAA and HbAS. However, for samples containing HbAC the immunoassay method showed relative positive biases of 8.4 and 10.4% at HbA_{1c} levels of 7 and 9%, respectively, such that the two methods would not be judged comparable according to NGSP guidelines.

CONCLUSIONS — The DCA 2000 HbA_{1c} immunoassay method showed significant positive bias in patients with HbC trait. One possible clinical implication of this overestimation is overly rigorous glycemic control with a concomitant increase in hypoglycemia. This may be especially important in certain ethnic populations, such as African-Americans, who have a relatively high prevalence of HbC trait.

The importance of good glycemic control in reducing the risk for the development and progression of chronic complications of diabetes has been demonstrated in a number of studies, including the Diabetes Control and Complications Trial (DCCT) (1). Glycemic control can be assessed by measurement of GHb, which correlates with the mean blood glucose concentration (2). A wide variety of methods that measure some form of GHb are available commercially. The National Glycohemoglobin Standardization Program (NGSP) was developed in an attempt to standardize these methods so that GHb results can be related to the candidate reference method used in the DCCT (3; additional information on the NGSP is available at http://www.missouri.edu/~diabetes/ngsp/). One area that has received some attention is the measurement of GHb in patients with Hb variants. Although relatively rare in Caucasians, in African-American populations the prevalence of sickle trait and HbC trait are 6–9 and 2–3%, respectively (4,5). The effects of Hb variants on several GHb methods have been investigated (6,7). The desire for rapid near-patient measurement of GHb has led to the development of rapid immunoassay methods for HbA_{1c}. The effects of Hb variants on these methods have not been carefully investigated. We compared two HbA_{1c} methods using samples containing HbAA, HbAC, and HbAS. Both methods are traceable to the candidate reference method used in the DCCT (3). One method (Bio-Rad Diamat) uses cation-exchange high-performance liquid chromatography (HPLC) to measure HbA_{1c} as a percentage of total HbA. The other method (Bayer DCA 2000) uses an immunoassay employing an antibody specific for the glycated NH_2-terminal of the Hb β-chain and can provide rapid results at the point of care.

RESEARCH DESIGN AND METHODS

Whole blood specimens in tubes containing EDTA were collected for routine HbA_{1c} determinations in diabetic patients and for Hb phenotype analysis. An attempt was made to collect approximately equal numbers of samples with HbAA (49 samples), HbAC (40 samples), and HbAS (43 samples) at each HbA_{1c} concentration. Samples with Hb variants were identified either by comparison of retention times on the Diamat system to known retention times for HbA, HbC, or HbS or by agarose gel electrophoresis. The percentage of each variant hemoglobin was determined on the Diamat system. Whole blood samples were stored at 4°C for up to 7 days. If analysis was anticipated to be delayed past this time, samples were stored at −70°C per vendor recommendations. The Diamat analyzer (Bio-Rad Clinical Labs, Hercules, CA) and the DCA 2000 analyzer (Bayer Diagnostics, Elkhart, IN) were used according to the manufacturers' instructions. Two levels of quality-control materials obtained from each manufacturer were run singly with each batch of samples (10 runs for the DCA 2000 and 12 runs for the Diamat). The routine time parameters for the Diamat

From the Department of Pathology (W.L.R., M.M.) and the Department of Internal Medicine (C.B.C.), University of Mississippi Medical Center, Jackson, Mississippi.
Address correspondence and reprint requests to William L. Roberts, MD, PhD, Department of Pathology, University of Mississippi Medical Center, 2500 North State St., Jackson, MS 39216. E-mail: wroberts@pathology.umsmed.edu.
Received for publication 29 August 1997 and accepted in revised form 18 February 1998.

Abbreviations: DCCT, Diabetes Control and Complications Trial; HPLC, high-performance liquid chromatography; NGSP, National Glycohemoglobin Standardization Program.

Downloaded from http://ada.silverchair.com/care/article-pdf/21/6/983/585918/21-6-983.pdf by guest on 17 April 2024

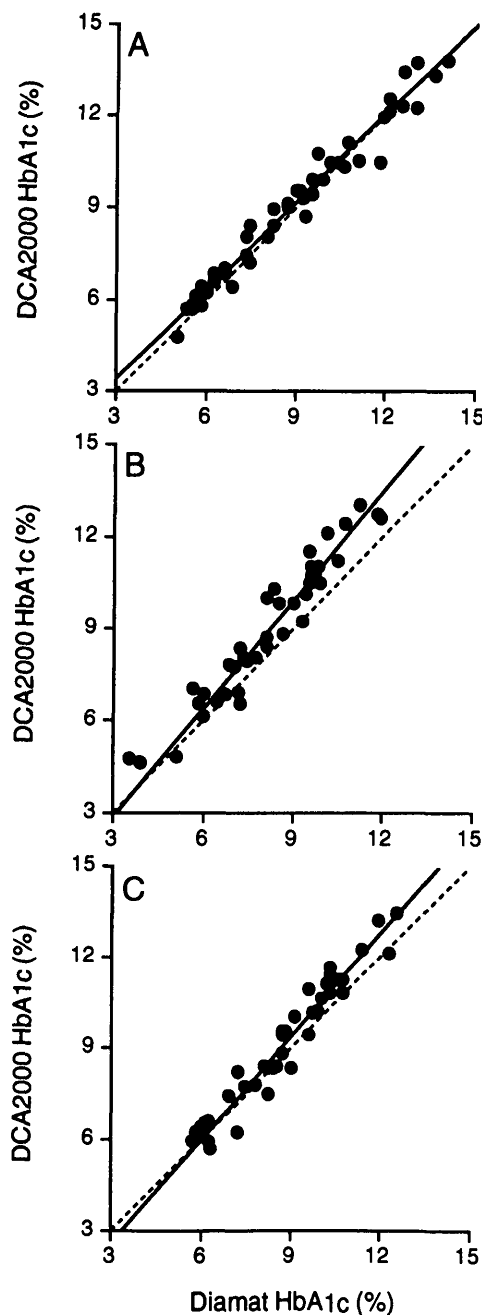

Figure 1—*Comparison of HbA_{1c} results obtained by Diamat and DCA 2000 analyzers from patients with HbAA, HbAS, and HbAC. Blood samples from patients with HbAA, HbAS, and HbAC were collected in EDTA and analyzed for HbA_{1c} on the Diamat and DCA 2000 analyzers. The results were compared by Deming regression analysis (—). --- represents an ideal comparison with a slope of 1.00 and an intercept of 0.0. Results from patients with HbAA are shown in A (n = 49, slope = 0.96 ± 0.02, intercept = 0.54 ± 0.22, r = 0.98, Sy/x = 0.46). Results from patients with HbAC are shown in B (n = 40, slope = 1.17 ± 0.05, intercept = −0.61 ± 0.45, r = 0.96, Sy/x = 0.70). Results from patients with HbAS are shown in C (n = 43, slope = 1.14 ± 0.04, intercept = −0.82 ± 0.37, r = 0.97, Sy/x = 0.54).*

were buffer 1 from 0 to 0.8 min, buffer 2 from 0.8 to 2.2 min, and buffer 3 from 2.2 to 3.8, with a total run time of 5.4 min. Samples with HbAC were rerun on the Diamat using an extended program with the same parameters as the routine program for buffers 1 and 2. Buffer 3 was run from 2.2 to 4.8 min, and the total run time was 6.4 min. This extended run time provided adequate resolution between HbA and HbC_{1c} to allow accurate quantitation of HbA.

Data analysis

EP Evaluator release 3 software (David G. Rhoads, Kennett Square, PA) was used for method comparisons by Deming regression analysis. Estimates of average bias and 95% CIs for bias estimates were also made using this software.

RESULTS — The precision of each assay was assessed using quality-control materials run with patient specimens. For the DCA 2000, the mean HbA_{1c} values for levels 1 and 2 were 5.8 and 11.6%, and the coefficients of variation were 2.7 and 4.9%, respectively (*n* = 10). For the Diamat, the mean HbA_{1c} values for levels 1 and 2 were 5.3 and 10.2%, and the coefficients of variation were 2.8 and 3.4%, respectively (*n* = 12). HbA_{1c} results from the Diamat and DCA 2000 analyzers were compared using samples from three groups of patients (Fig. 1). The first group of samples were from patients with HbAA and served as controls. Samples from patients with two groups of Hb variants (AC and AS) were also compared. The mean percentage of HbC in samples with HbAC was 38% (range 29–41) and the mean percentage HbS in samples with HbAS was 38% (25–41). The results of these two DCCT-traceable methods compared very well when samples with HbAA were analyzed (Fig. 1*A*). Although the correlation coefficients for HbAA, HbAC, and HbAS were all ≥0.96, the comparability between methods with samples containing HbAC (Fig. 1*B*) and HbAS (Fig. 1*C*) was not as good with the DCA 2000, showing a positive bias compared with the Diamat method. Estimates of the average bias of the DCA 2000 with 95% CIs were calculated for HbA_{1c} levels between 5 and 14% in 1% increments for each of the three Hb types (Table 1). The average relative bias of the DCA 2000 method compared with the Diamat method was estimated from Deming regression analysis and plotted as a function of HbA_{1c} (Fig. 2). The relative bias was not linear for samples with HbAA, HbAC, or HbAS. In samples with HbAS, the average relative bias of the DCA 2000 method exceeded 5% at HbA_{1c} levels of ≥10%. However, the lower limit of the 95% CI of the estimated bias for HbA_{1c} levels between 5 and 14% was within the ± 5% limit set by the NGSP (3). In contrast, for samples with HbAC, the average relative bias of the DCA

Downloaded from http://ada.silverchair.com/care/article-pdf/21/6/983/585918/21-6-983.pdf by guest on 17 April 2024

Table 1—*Estimates of DCA 2000 average HbA_{1c} values with 95% CIs and mean blood glucose values for samples containing HbAA, HbAC, and HbAS*

Diamat HbA_{1c} value	HbAA	HbAC	HbAS
5.0 [94]	5.33 (5.11–5.55) [104]	5.25 (4.85–5.65) [102]	4.85 (4.50–5.20) [89]
6.0 [125]	6.29 (6.10–6.47) [134]	6.42 (6.11–6.73) [138]	5.99 (5.71–6.26) [124]
7.0 [156]	7.24 (7.09–7.40) [163]	7.59* (7.35-7.84) [174]	7.12 (6.91–7.34) [159]
8.0 [187]	8.20 (8.07–8.33) [193]	8.76* (8.55–8.98) [210]	8.26 (8.09–8.43) [195]
9.0 [218]	9.16 (9.03–9.29) [222]	9.94* (9.70–10.17) [247]	9.39 (9.23–9.55) [230]
10.0 [248]	10.12 (9.98–10.26) [252]	11.11* (10.81–11.40) [283]	10.53 (10.34–10.72) [265]
11.0 [279]	11.07 (10.91–11.24) [281]	12.28* (11.90–12.65) [319]	11.66 (11.41–11.91) [300]
12.0 [310]	12.03 (11.83–12.23) [311]	13.45* (12.98–13.92) [355]	12.80 (12.48–13.12) [335]
13.0 [341]	12.99 (12.75–13.23) [341]	14.62* (14.06–15.19) [391]	13.93 (13.54–14.33) [370]
14.0 [372]	13.95 (13.66–14.23) [370]	15.79* (15.13–16.46) [427]	15.07 (14.60–15.54) [405]

Data are % [mean blood glucose] or % (95% CI) [mean blood glucose]. The average DCA 2000 HbA_{1c} values were estimated using Deming regression analysis. Mean blood glucose (MBG) values are given in milligrams per deciliter and were calculated using the equation $MBG = 30.9 \times HbA_{1c} - 60.6$ (2). *The 95% CI of this value exceeds the 5% bias limit set by the NGSP guidelines for method comparability.

2000 method ranged from 5 to 13% for HbA_{1c} levels between 5 and 14%. Furthermore, at HbA_{1c} levels of ≥7%, the lower limit of the 95% CI of the estimated bias exceeded the 5% NGSP limit, indicating that the two methods were not comparable for this group of samples. Mean blood glucose values for Diamat and corresponding DCA 2000 results were also estimated using a formula derived from data collected in the DCCT (Table 1) (2).

To validate the accuracy of the Diamat extended program for HbA_{1c} determinations, we analyzed an additional group of 43 samples containing HbAA and HbAS with HbA_{1c} values from 5.1 to 16.0% using both the standard and extended programs. Deming regression analysis of the standard versus the extended program gave a slope of 0.996, an intercept of −0.12, a correlation coefficient of 0.9997, and an SE of the estimate of 0.07. Additional verification of the accuracy of the Diamat method for samples with HbAC was obtained by comparisons with the A_{1c} Plus (Tosoh Medics, Foster City, CA), a high resolution HPLC method that was recently certified as traceable to the DCCT candidate reference method by the NGSP. Comparisons of the Diamat and A_{1c} Plus methods using samples with HbAA and HbAC revealed nearly identical slopes and intercepts for both groups of samples (8).

CONCLUSIONS — Good glycemic control, as assessed by measurement of HbA_{1c}, is important in reducing the complications of diabetes (1). The accuracy of HbA_{1c} measurements and the traceability of analytical methods for GHb measurement to the method used in the DCCT are critical to appropriate clinical management of diabetic patients. The NGSP has three criteria for documentation of traceability to the candidate reference method used in the DCCT (3). One of these states that total imprecision must not be statistically significant at >5%. In our limited tests, both methods appear to satisfy this criterion. Another criterion, which is derived from the National Committee for Clinical Laboratory Standards guidelines, states that the 95% CI for predicted bias should overlap the ±5% range of the Secondary Reference Laboratory at two GHb levels (6 and 9% HbA_{1c}) (9). In our laboratory, a comparison of the Diamat and DCA 2000 methods met this criterion for samples with HbAA and HbAS. However, when analyzing samples with HbAC, the DCA 2000 method demonstrated a positive bias relative to the Diamat that exceeded the acceptable bias limit at HbA_{1c} levels >7%.

Estimates of mean blood glucose values were made using the mean bias estimates for the DCA 2000 and a recently derived equation from the DCCT (2). An average patient with HbC trait and an HbA_{1c} level of 6% by the Diamat would have a corresponding HbA_{1c} of 6.4% on the DCA 2000, with a consequent overestimate of the mean blood glucose by 10% (138 vs. 125 mg/dl), and for a Diamat HbA_{1c} of 7%, the DCA 2000 would overestimate the mean blood glucose by 12% (174 vs. 156 mg/dl). These overestimates of HbA_{1c} and mean blood glucose might lead to unnecessary attempts to tighten glycemic control with its attendant risk of hypoglycemia.

Although the use of HbA_{1c} measurements to screen for diabetes is not recommended, several reports have explored the feasibility of its use in this role (10). Given the large positive bias of the DCA 2000 method in patients with HbC trait (relative bias 8.4% at an HbA_{1c} of 7.0%), significant problems are likely to arise if the DCA 2000 method is used for this purpose in a population with a high prevalence of HbC trait.

The positive bias of immunoassay results relative to HPLC results in patients with HbAC may be understood as follows. The HPLC method resolves and quantitates HbA_{1c}, HbA_{1a}, HbA_{1b}, and HbA directly. HbA_{1c} is then expressed as percentage of total A. The antibody used in the immunoassay method recognizes the glycated NH_2-terminal of the HbA and presumably also HbC, HbS, and other variant Hb β-chains. This method also measures total Hb and calculates an HbA_{1c} as the glycated NH_2-terminal of the Hb β-chain divided by total Hb. One explanation for the discrepancy between the two methods could be the preferential nonenzymatic glycation of the NH_2-terminal of the β-chain of HbC (11,12). Our data are consistent with preferential glycation occurring at the NH_2-terminal of the β-chain of HbC because of the specificity of the DCA 2000 antibody for this site. If the positive bias on samples with HbAC is due to an enhanced rate of NH_2-terminal glycation rate of the β-chain of HbC, then other immunoassay methods would be expected to be similarly affected. An earlier study of glycohemoglobin reference intervals in nondiabetic patients revealed that patients with HbAC had significantly higher reference interval

Downloaded from http://ada.silverchair.com/care/article-pdf/21/6/983/585918/21-6-983.pdf by guest on 17 April 2024

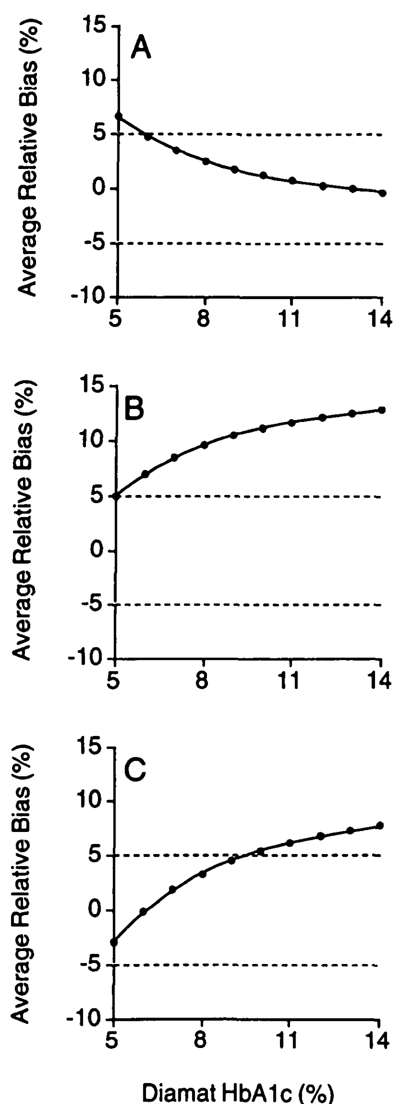

Figure 2—*Average relative bias of HbA_{1c} results from the DCA 2000 method for samples with HbAA, HbAS, and HbAC. The estimated DCA 2000 average HbA_{1c} values from Table 1 were used to estimate the relative bias in percent of the DCA 2000 using the equation (DCA 2000 − Diamat)/Diamat × 100. The relative bias was plotted against the Diamat HbA_{1c}. The results for HbAA are shown in A, HbAC in B, and HbAS in C. The dashed lines indicate the ± 5% error limits from the NGSP.*

mean values than patients with HbAA by two affinity chromatography methods (Helena Quick Column and Isolab Glyc-Affin) and one immunoassay method (Boehringer Tina Quant) (7). Another possibility is that the substitution of lysine for glutamic acid at position 6 of the β-chain in HbC could alter the immunoreactivity of the glycated NH_2-terminal such that the antibody in the DCA 2000 method recognizes HbC_{1c}, but its cross-reactivity is not linear, and higher concentrations of HbC_{1c} show a positive bias. Interestingly, in HbS, valine is substituted for glutamic acid at the same position, yet the DCA 2000 does not demonstrate a significant positive bias in HbAS samples. Whatever mechanism is responsible for the observed positive bias of the DCA 2000 method relative to the Diamat method, the data clearly indicate that these two methods do not give comparable HbA_{1c} values in patients with HbAC.

An accurate assessment of GHb is important for management aimed at preventing the long-term complications of diabetes. Our data suggest that when evaluating glycemic control in the African-American population, a subgroup with a high rate of diabetes and Hb variants, that the analytical method used to determine HbA_{1c} in patients with HbC trait may be important to properly titrate therapy for hyperglycemia.

References

1. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
2. Goldstein DE, Wiedmeyer H-M, Little RR, Varagas V, Nair SS, Reid J: Relationship between glycohemoglobin (GHB) and mean blood glucose (MBG) in the Diabetes Control and Complications Trial (DCCT) (Abstract). *Diabetes* 46 (Suppl. 1):8A, 1997
3. NGSP Steering Committee: Implementation of the National Glycohemoglobin Standardization Program (NGSP) [Abstract]. *Diabetes* 46 (Suppl. 1):151A, 1997
4. Schneider RG, Hightower B, Hosty TS, Ryder H, Tomlin G, Atkins R, Brimhall B, Jones RT: Abnormal hemoglobins in a quarter million people. *Blood* 48:629–637, 1976
5. Meyer, LM, Adams JG 3rd, Steinberg MH, Miller IE, Stokes N: Screening for sickle cell trait: the Veterans Administration National Sickle Cell Program. *Am J Hematol* 24:429–432, 1987
6. Weykamp CW, Penders TJ, Muskiet FAJ, van der Slik W: Influence of hemoglobin variants and derivatives on glycohemoglobin determinations, as investigated by 102 laboratories using 16 methods. *Clin Chem* 39:1717–1723, 1993
7. Weykamp CW, Martina WV, van der Dijs FPL, Penders TJ, van der Slik W, Muskiet FAJ: Hemoglobin S and C: reference values for glycohemoglobin in heterozygous, double heterozygous and homozygous subjects, as established by 13 methods. *Clin Chim Acta* 231:161–171, 1994
8. Ward KM, Chiasera JM, Roberts WL: Greater variability in glycohemoglobin test results in patient samples with hemoglobin variants: a comparison of four test systems (Abstract). *Clin Chem.* In press
9. National Committee for Clinical Laboratory Standards: Method comparison and bias estimation using patient samples: tentative guideline. Villanova, PA, NCCLS, 1993 (NCCLS publication EP9-T)
10. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus: Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 20:1183–1197, 1997
11. Tegos C, Blume K, Johnson C, Beutler E: Glycosylated minor C, D, and E hemoglobins. *Biochem Med* 26:121–125, 1981
12. Goujon R, Thivolet C: Glycation of hemoglobin C in the heterozygous state in diabetic patients (Letter). *Diabetes Care* 17:247, 1994

Downloaded from http://ada.silverchair.com/care/article-pdf/21/6/983/585918/21-6-983.pdf by guest on 17 April 2024