concentrations with age, our results nevertheless represent a useful set of reference values in children.

We thank Marisa Punzo, Angela Sglavo, and Bianca Tesone for excellent technical assistance in the assay procedure.

References


Effects of Hemoglobin C and S Traits on Seven Glycohemoglobin Methods, Elizabeth L. Frank,1 Linda Moulton,2 Randie R. Little,3 Hsiao-Mei Wiedmeyer,3 Curt Rohlfing,3 and William L. Roberts1* (1 University of Utah, Department of Pathology, Salt Lake City, UT 84108; 2 ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT 84108; 3 University of Missouri-Columbia School of Medicine, Departments of Child Health and Pathology, Columbia, MO 65212; * address correspondence to this author at: ARUP Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108; fax 801-584-5207, e-mail william.roberts@arup-lab.com)

Glycohemoglobin (gHb) is a marker of long-term glycemic control that has been shown to correlate with complications of diabetes mellitus (1). The National Glycohemoglobin Standardization Program (NGSP) was established to standardize gHb results so that clinical laboratory results are comparable to those reported by the Diabetes Control and Complications Trial (2,3). Previous studies have shown that some gHb methods yield inaccurate results with samples heterozygous for hemoglobin (Hb) C or Hb S (4–6). At least 10% of black Americans have either Hb C or S trait, and there were 19 million black Americans over age 19 in 1990 (7–10). The prevalence of diabetes is estimated to be 5.1% of the adult population, with the rate for non-Hispanic blacks being 1.6-fold higher than that of non-Hispanic whites (11). It is therefore probable that at least 150 000 Americans with

Fig. 1. Confidence intervals for mean values in boys and girls.

Values in ng/L.

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diabetes have Hb C or S trait. Here, we investigate the measurement of gHb in specimens containing Hb C or S trait using eight gHb methods currently in clinical use.

Whole blood samples from individuals homozygous for Hb A (n = 43) and heterozygous for Hb C or S (n = 43 and 61, respectively) were collected in EDTA tubes. Hb variants were identified by their characteristic Variant A1c HPLC chromatograms (Bio-Rad Laboratories). Aliquots of these samples containing between 4% and 14% Hb A1c (NGSP Hb A1c evaluation range) were stored refrigerated (2–8 °C) and analyzed within 10 days of collection. This study was approved by the Institutional Review Board of the University of Utah.

The CLC 330 gHb analyzer (Primus Corporation) was operated at the University of Missouri. This method was chosen as the comparison method for this study because results from boronate affinity chromatography should not be influenced by the presence of Hb variants (12–15). The following methods/instruments were evaluated: the A1c 2.2 Plus™ Glycohemoglobin Analyzer ( Tosoh Medics), the Diamat™ Analyzer System and a Variant™ Hemoglobin Testing System (Bio-Rad Laboratories), the IMx® Glycated Hemoglobin assay (Abbott Laboratories), the DCA 2000 Analyzer System (Beckman Instruments), Tina-quant II on an Hitachi 717 analyzer (Roche Diagnostics), and the CLC 330 Analyzer (Bayer Corporation, Elkhart, IN).

To eliminate suspected carryover when samples containing Hb C trait were analyzed by the A1c 2.2 Plus method, three samples homozygous for Hb A were analyzed but not reported after each sample containing Hb C trait. Diamat and CLC 330 HPLC analyses were performed in NGSP Network Laboratories. In these laboratories, in-house calibrator materials and assigned values were used. For all other methods, samples were analyzed according to the manufacturers’ instructions. For samples containing Hb AC, the Diamat 10-min extended program was used. A subset of samples with Hb AS (n = 10) was analyzed by Diamat HPLC using two different column series (P and R); all other samples were analyzed using a P-series column.

For each test method, results for each group of samples (Hb AA, Hb AC, and Hb AS) were compared with results from the comparison method (CLC 330). An overall test for coincidence of two least-squares linear regression lines was performed using SAS software (SAS Institute) to determine whether the presence of Hb C or S trait caused a statistically significant difference (P < 0.01) in results relative to the comparison method. The presence of Hb C produced statistically significant differences for all methods tested except the A1c 2.2 Plus (P = 0.014) and the Variant A1c (P = 0.411). The presence of Hb S trait produced statistically significant differences for all methods except the IMx (P = 0.072).

Deming regression analysis was performed using EP Evaluate, release 3, software (David G. Rhoads, Kennett Square, PA) to determine whether the presence of Hb C and S traits produced clinically significant effects. Given the Diabetes Control and Complications Trial Reference Method upper limit of normal of 6%, and the American Diabetes Association goal and action limits of 7% and 8%, respectively, Hb A1c concentrations of 6% and 9% were chosen as important evaluation limits. Given the need to clearly distinguish the difference among 6%, 7%, and 8% Hb A1c, a >10% deviation (0.6% at 6% Hb A1c and 0.9% at 9% Hb A1c) compared with the Hb AA sample group was used to define a clinically significant difference. No clinically significant interference attributable to Hb AC or Hb AS was seen with the A1c 2.2 Plus, DCA 2000, and Tina-quant II methods (Table 1). The Diamat and Variant A1c methods showed clinically significant interference from Hb AS but not from Hb AC. Both Hb AC and Hb AS produced clinically significant interferences for the Diatrac and IMx methods, although the difference observed with Hb S with the IMx method was not statistically significant. Box plots for each group of samples and for each method are shown in Fig. 1. Of note is the increased scatter observed for the A1c 2.2 Plus, Diatrac, and Variant A1c methods for samples containing Hb C or S trait compared with Hb AA samples.

Our data for Hb AS samples with the Diamat method were not consistent with previous results (5, 6). One difference between the current study and these two previous reports was that an 8-min program was used in the current study, whereas a 5-min program was used previously. Another potential difference was the series of column and reagents used. This information was not provided for either of the previous studies. The R series replaced the P series in March 1999; this series contained a new lot of packing material, which provided improved peak resolution. To determine whether differences between these two series could also contribute to variability with Hb variants, a subset of samples containing Hb AS was analyzed using two different Diamat column series. The P-series column gave higher results with Hb AS, which was more pronounced in the lower Hb A1c range (slope = 0.89; intercept = 2.16); R-series column results

<table>
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<th>Table 1. Average differences from the comparison method for samples containing either Hb C or S traits.a</th>
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<td><strong>Hb C trait</strong></td>
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<tr>
<td>Method</td>
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<tr>
<td>A1c 2.2 Plus</td>
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<tr>
<td>DCA 2000</td>
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<td>Diamat</td>
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<td>Tina-quant II</td>
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<td>Variant A1c</td>
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a Deming regression analysis was performed using the CLC 330 as the comparison method. The average differences of each of the other seven methods at clinical decision cutoffs of 6% and 9% were calculated for each Hb trait. To correct for immethorod calibration differences, the mean difference for homozygous Hb A samples was subtracted from that calculated for samples containing Hb C or Hb S trait.

b Both statistically significant (P < 0.01) and clinically significant (>0.6% or >0.9% at 6% and 9% Hb A1c, respectively) differences were found.
with Hb AS, on the other hand, were lower at higher Hb A1c concentrations (slope = 0.74; intercept = 1.77).

One explanation for the overestimated Hb A1c results observed for Hb S trait with the Diamat P column and Variant A1c methods and the underestimated results observed with the Diamat R column may be that Hb A0 and Hb S0 are not well resolved from each other. This could lead to underestimation of Hb A0 and consequent overestimation of the percentage of Hb A1c or vice versa. Overestimation of Hb A1c is most apparent at concentrations <8%. Clinically, this may lead to overly rigorous glycemic control with a consequent increase in hypoglycemic episodes. This hypothesis requires formal testing in a clinical outcome study.

In Hb C, lysine is substituted for glutamic acid at position 6 in the β chain; in Hb S valine is substituted at the same position (4). In immunoassay methods, these amino acid substitutions at position 6 may alter the shape of the protein sufficiently to change its binding characteristics with the reagent antibody (15). This may explain the small positive bias in the DCA 2000 assay and small negative bias in the Tina-quant II assay. The DCA 2000 was previously reported to give falsely increased results for Hb AC (5, 6). However, the Diamat, not a boronate affinity method, was used as the comparison method.

The only electrophoretic method evaluated in this study was the Diatrac system. Significant underestimation of the percentage of Hb A1c was observed for samples containing either Hb C or S trait, with samples containing Hb S trait being the most affected. In this system, Hb A1c is resolved from Hb C1c and Hb S1c (Hb C and Hb S glycated at the NH2 terminus of the Hb β chain), but Hb A0 is not resolved from Hb C0 and Hb S0. This leads to an underestimation of the percentage of Hb A1c, that is proportional to the amount of Hb C0 or Hb S0 present in the sample. Samples containing Hb AC or Hb AS should not be analyzed by this method.

The IMx method uses a boronate affinity reagent coupled with an ion-capture reagent to measure total gHb. One possible explanation for the overestimation of Hb A1c is that charge interactions between the lysine residue at position 6 of the Hb C β chain and the polyanion reagent might lead to capture of nonglycated Hb by the polycation capture matrix with consequent overestimation of gHb.

In conclusion, the presence of Hb C or S trait can produce clinically significant differences in gHb results for some methods. These differences may, in some cases, be dependent on the lot of reagents used. More careful attention to the effects of Hb variants on the results of gHb methods is necessary to ensure accurate results for the >150,000 Americans who have a Hb variant and diabetes mellitus. In addition, more detailed information about the effects of common Hb variants, including Hb C and S trait, should be included in the package insert of each commercially available gHb method.

A DCA 2000 instrument and reagents were provided by Bayer Diagnostics. Tina-quant II reagents were provided by Roche Diagnostics. IMx reagents were provided by Abbott Diagnostics. Beckman Coulter provided all results for the Diatrac method. This work was supported in part by the ARUP Institute for Clinical and Experimental Pathology. We thank Dr. Tom Lohmann (Ochsner Foundation Hospital, New Orleans, LA) and Marilyn Carpenter (University of Mississippi Medical Center, Jackson, MS) for providing samples containing Hb C and S trait, and Drs. Kory Ward-Cook and Jim Noffsinger for critical review of this manuscript.

References
Nonparametric Estimation of Reference Intervals by Simple and Bootstrap-based Procedures, Kristian Linnet

In recent years, increasing interest has arisen in nonparametric estimation of reference intervals. The IFCC recommendation focuses on the nonparametric procedure, and the NCCLS guideline on reference interval estimation deals exclusively with the nonparametric approach (1, 2). The mentioned reports are based on the simple nonparametric approach, taking as a basis the sorted sample values. In addition to this basic approach, modern computer-based procedures have been introduced, which have made it possible to attain slightly increased precision for the nonparametric approach by applying resampling methods, weighted percentile estimation, or smoothing techniques (3, 4). In the present report, both the simple nonparametric reference interval estimation procedure and the resampling (bootstrap) principle were studied using simulations based on distribution types that should be relevant for clinical chemistry, i.e., gaussian and skewed distributions.

According to the procedure recommended by the IFCC and NCCLS, the observations are ranked according to size, and the 2.5 and 97.5 percentiles are obtained as the 0.025 (n + 1) and 0.975 (n + 1) ordered observations (1, 2). If the estimated rank values are not integers, then linear interpolation is carried out. In the statistical literature, various modifications of the computation procedure have been considered (5–7). Here the traditional one used in clinical chemistry as outlined above (called method I) is compared with an alternative (called method II): \( p/100 \times n + 0.5 \), where \( p \) indicates the percentile (6). For the 2.5 and 97.5 percentiles, method II yields the 0.025n + 0.5 and 0.975n + 0.5 ordered values, respectively. In the following, the above-mentioned calculation principles are referred to as “simple” procedures (IS or IIS) as opposed to “bootstrap” modifications described below (IB or IIB).

The bootstrap principle consists of repeated random resampling of the original observations with replacement, which is performed by a computer (8, 9). Each of the original observations is assigned the same probability of being resampled, i.e., \( 1/n \). For each set of \( n \) resampled values, percentile estimates are computed as usual. After repetition of the procedure a large number of times, e.g., 50–500 times, the bootstrap estimates are obtained as the means of these percentile estimates. In the present study, 100 replications were carried out.

A gaussian population distribution was considered as a basis (Fig. 1, top left panel). A mean (\( \mu \)) of zero and a standard deviation (\( \sigma \)) of 1 were selected as parameters, i.e., corresponding to the standard gaussian distribution. The true 2.5 and 97.5 percentile values for a gaussian distribution are \( \mu \pm 1.96 \times \sigma \), respectively, i.e., -1.96 and +1.96 for the standard gaussian distribution.

The skewed distribution was generated on the basis of standard gaussian distributed values subjected to the inverse of the Manly exponential transformation \( y = [\log (1 + ky)]/k \) with a selected parameter producing a coefficient of skewness of 1.5 (Fig. 1, top right panel) (10, 11). This degree of skewness may, for example, be observed for reference value distributions of serum concentrations of enzymes (11).

The root mean squared error (RMSE) of percentile estimates represents an overall measure of bias and imprecision for a given procedure and allows a ranking of the studied procedures. The bottom left panel of Fig. 1 displays a comparison of the RMSE values of simple and bootstrap modifications of methods I and II with regard to the upper percentile for the gaussian distribution (same as for the lower percentile). The IIS procedure clearly outperforms the IS method, and the bootstrap version of method II has the lowest RMSE of all procedures for all sample sizes. The same ranking of the procedures is valid for estimation of the upper percentile of the skewed distribution (Fig. 1, bottom right panel). In general, the differences between the procedures are most pronounced at low to moderate sample sizes. At a sample size of 40, the RMSE of the bootstrap version of method II is 30% (gaussian distribution) or 42% (skewed distribution) lower than that of the IS procedure. The differences are 8% and 7% at a sample size of 500.

A valid statistical estimation method should provide a realistic estimate of the uncertainty associated with the procedure, e.g., expressed as a 90% confidence interval. For the simple estimation procedures (IS and IIS), a 90% confidence interval for the reference interval limits may be derived from the sorted sample values when \( n \) is at least 120 (1). Simulations showed that for both the gaussian and the skewed distributions, the actual coverage...