Effects of Hemoglobin (Hb) E and HbD Traits on Measurements of Glycated Hb (HbA1c) by 23 Methods

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BACKGROUND: Glycohemoglobin (GHB), reported as hemoglobin (Hb) A1c, is a marker of long-term glycemic control in patients with diabetes mellitus. The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study demonstrated conclusively that risks for complications are directly related to glycemic control, as measured by HbA1c (1, 2). Many diabetes organizations worldwide recommend specific HbA1c targets in terms of DCCT/United Kingdom Prospective Diabetes Study HbA1c. The National Glycohemoglobin Standardization Program was established to standardize GHB/HbA1c results so that clinical laboratory results are comparable to those reported by the DCCT. However, the accuracy of several HbA1c methods can be adversely affected by the presence of Hb variants (3), and the National Glycohemoglobin Standardization Program does not include evaluation of interferences as part of the certification program. The most common Hb variants worldwide are HbS, HbE, HbC, and HbD. Unlike HbS and HbC traits, for which most of the commonly used HbA1c methods have already been, and continue to be, evaluated (4–6) few data are available on the accuracy of HbA1c measurement in the presence of HbE or HbD traits. Therefore we evaluated the effects of HbE and HbD trait on 23 commercial GHB methods using the Primus boronate affinity HPLC assay (ultra2) as the comparison method.

METHODS: We evaluated 23 HbA1c methods; 9 were immunoassay methods, 10 were ion-exchange HPLC methods, and 4 were capillary electrophoresis, affinity chromatography, or enzymatic methods. An overall test of coincidence of 2 least-squares linear regression lines was performed to determine whether the presence of HbE or HbD traits caused a statistically significant difference from HbAA results relative to the boronate affinity HPLC comparative method. Deming regression analysis was performed to determine whether the presence of these traits produced a clinically significant effect on HbA1c results with the use of ±10% relative bias at 6% and 9% HbA1c as evaluation limits.

RESULTS: Statistically significant differences were found in more than half of the methods tested. Only 22% and 13% showed clinically significant interference for HbE and HbD traits, respectively.

CONCLUSIONS: Some current HbA1c methods show clinically significant interferences with samples containing HbE or HbD traits. To avoid reporting of inaccurate results, ion-exchange chromatograms must be carefully examined to identify possible interference from these Hb variants. For some methods, manufacturers’ instructions do not provide adequate information for making correct decisions about reporting results.

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Glycohemoglobin (GHB),8 reported as hemoglobin (Hb) A1c, is a marker of long-term glycemic control in patients with diabetes mellitus. The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study demonstrated conclusively that risks for complications are directly related to glycemic control, as measured by HbA1c (1, 2). Many diabetes organizations worldwide recommend specific HbA1c targets in terms of DCCT/United Kingdom Prospective Diabetes Study HbA1c. The National Glycohemoglobin Standardization Program was established to standardize GHB/HbA1c results so that clinical laboratory results are comparable to those reported by the DCCT. However, the accuracy of several HbA1c methods can be adversely affected by the presence of Hb variants (3), and the National Glycohemoglobin Standardization Program does not include evaluation of interferences as part of the certification program. The most common Hb variants worldwide are HbS, HbE, HbC, and HbD. Unlike HbS and HbC traits, for which most of the commonly used HbA1c methods have already been, and continue to be, evaluated (4–6) few data are available on the accuracy of HbA1c measurement in the presence of HbE or HbD traits. Therefore we evaluated the effects of HbE and HbD trait on 23 commercial GHB methods using the Primus boronate affinity HPLC assay (ultra2) as the comparison method.

HbE contains a substitution of lysine for glutamic acid at position 26 of the β-globin chain. HbE is the

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Nonstandard abbreviations: GHB, glycated hemoglobin; Hb, hemoglobin; DCCT, Diabetes Control and Complications Trial; SRL, Secondary Reference Laboratory.
second most prevalent Hb variant worldwide and is found primarily in people from Southeast Asia; prevalence is 30%–40% in some parts of Thailand, Cambodia, and Laos (7). Owing to an influx of immigrants from Southeast Asia in recent years, HbE is now encountered quite commonly in the US. HbD Punjab (also called HbD Los Angeles), hereafter referred to as HbD, contains a substitution of glutamine for glutamic acid at position 121 of the \( \beta \)-globin chain. HbD Punjab is found most commonly in the Punjab region of India (2%–3% prevalence in Sikhs in Punjab) and is also encountered in the US (8).

**Materials and Methods**

**SAMPLES**

This study was approved by the ethics review committee at DynaLIFE Dx in Edmonton, Canada, where the samples originated. Whole blood samples from individuals homozygous for HbA (n = 49) and for HbE or HbD trait (HbAE, HbAD) (n = 42 for each trait) were collected in EDTA-containing tubes. After routine clinical testing had been completed, the samples previously identified as having HbD Punjab or HbE traits by use of the Bio-Rad Beta Thalassemia HPLC system and Sebia Hydrasys electrophoresis at both alkaline and acid pH were shipped on cold packs to the Diabetes Diagnostic Laboratory at the University of Missouri (Columbia, MO). Several small aliquots were made from each sample and stored at \(-70 \, ^{\circ}\text{C} \) until they were shipped on dry ice to various sites for analysis.

**Assay methods.** Samples were analyzed by the following instruments/methods grouped by method type: Immunoassay methods used were AlcNow (Metrika/Bayer), Synchron UniCel DxC (Beckman Coulter), Dimension RxL (Dade Behring), Au400 (Olympus Diagnostics), Vitros (Ortho-Clinical Diagnostics), HbA\(_{1c}\) (Pointe Scientific) on Modular P, Integra 800 Gen2 and Tina-quant on Hitachi 917 (Roche Diagnostics), and DCA2000 (Siemens Medical Solutions). Ion-exchange HPLC methods used were D-10 (short and extended programs), Variant, Variant II Turbo, and Variant II NU (Bio-Rad Laboratories); HA8160 Diabetes mode and HA8160 Thalassemia Program (TP) mode (A. Menarini Diagnostics); and Alc 2.2 Plus, G7 (Variant Analysis Mode), and G8 (Variant Analysis Mode) (Tosoh Biosciences). Capillary electrophoresis, enzyme, and boronate affinity HPLC methods used were CE on PACE 5000 (Analis, Beckman Coulter), Direct Enzymatic HbA\(_{1c}\) (Diazyme Laboratories) on Modular P (Roche Diagnostics), Afinion (Axis-Shield), and PDQ (Primus Diagnostics).

The Primus ultra\(^2\) HPLC method was used as the comparison method because results from boronate affinity chromatography are not expected to be influenced by the presence of Hb variants (9–13). The G7, Tina-quant on Hitachi 917, ultra\(^2\), CE, and HA8160 diabetes modes are National Glycohemoglobin Standardization Program Secondary Reference Laboratory (SRL) methods (SRL7, SRL4, SRL3, ESRL3, and ESRL7, respectively).

**Data Analysis.** For each test method, results obtained for each type of sample (homozygous HbA, HbE trait, and HbD trait) were compared to those we obtained using the ultra\(^2\) comparison method. An overall test of coincidence of 2 least-squares linear regression lines was performed using SAS software (SAS Institute) to determine whether the presence of HbE or HbD trait caused a statistically significant difference \((P < 0.01)\) in results relative to the comparison method. Deming regression analysis was performed to determine whether the presence of HbE or HbD trait produced a clinically significant effect on HbA\(_{1c}\) results. Given recommendations by the American Diabetes Association of an upper reference limit of 6%, the American Diabetes Association goal of 7%, and the DCCT conventional group mean HbA\(_{1c}\) of approximately 9%, we chose HbA\(_{1c}\) evaluation limits of 6% and 9%. After correcting for possible calibration bias by comparing results from the homozygous HbA sample group, we evaluated method bias due to the presence of HbE or HbD trait, with a clinically significant difference being \(>10\%\) relative bias at 6% and 9% HbA\(_{1c}\) (i.e., 0.6% at 6% HbA\(_{1c}\) and 0.9% at 9% HbA\(_{1c}\)).

**Results**

Box plots for each group of samples and for each method, with indications of both statistical and clinical significance are shown in Fig. 1. Although statistically significant differences were found in more than half of the methods tested, only 5 of 23 and 3 of 23 showed clinically significant interferences for HbE and HbD traits, respectively. Table 1 lists the differences at 6% and 9% HbA\(_{1c}\) for each variant and for each method. There were no clinically significant differences seen for either HbE or HbD traits for any of the immunoassay methods tested, or for the PDQ, Afinion, Enzymatic method, CE method, Variant II NU, or D-10 short and extended programs. In addition, there were no clinically significant differences seen for HbD trait for the 2.2 Plus, G7, or G8. There were no clinically significant differences seen for the HbE trait for the Variant and the HA-8160 TP mode. The Variant II Turbo showed clinically significant interference from both HbE and HbD traits, as did the Menarini HA8160 diabetes mode. The HA-8160 TP mode does not quantitate HbA\(_{1c}\) in the presence of HbD trait.
In addition to the actual bias in the HbA1c result caused by the presence of these variant Hbs, it is important that any unacceptable results are detected and not reported. Fortunately, we found that results from samples with HbE and HbD traits do not show interference with HbA1c measurement by the immunoassay, enzymatic, and boronate affinity methods that were evaluated, because Hb variants are not discernable with these methods. It is not surprising that HbE and HbD traits do not interfere with immunoassay methods, given that the amino acid substitutions are far from the N-terminus of the β chain where HbA1c glycation and antibody binding occur. These results were consistent with a previous report (12).

In cases in which ion-exchange methods show clinically significant interferences, it is important to know whether following manufacturer instructions would prevent the reporting of unacceptable results. Unfortunately, some manufacturer instructions for acceptance of results by ion-exchange methods are not clearly stated, and some manufacturers do not provide sample chromatograms. Examples of chromatograms from such methods with clinically unacceptable results are shown in Supplemental Data Figs. 1–4, which are available at www.clinchem.org with the electronic version of this manuscript.

For the Tosoh 2.2 Plus, the manufacturer states that it is acceptable to report an “Hb Var” peak after HbA0; this would include a peak for the HbD trait. Our results confirm the acceptability of these manufacturer instructions. If there is an unidentifiable peak before the A0 peak (e.g., P00, P01), the result is not reportable; this is the case for HbE trait, and indeed our data show nonreportable results for HbAE. For the Tosoh G7, the manufacturer instructions say that any abnormal peak before HbA0 will cause erroneous HbA1c results; this includes a peak for the HbE trait. Our results confirm that such abnormal peaks are indeed not reportable. Because the HbD peak appears after the HbA0 peak, results showing this peak should be reportable; our study data confirm this. For the G8, the manufacturer instructions state that the HbA1c is reportable for HbD trait because the variant peak and P00 peaks elute after the A0 and their sum is not >55%. HbAE is stated to be
nonreportable because an extra peak appearing between the A1c and A0 peak. HbE and D trait chromatograms appear in the G8 instruction manual. The present results confirm the manufacturer’s instructions and show that HbD trait results are accurate, whereas HbE trait results are not.

For the Variant no information is provided by the manufacturer about whether or not HbE or HbD trait chromatograms are acceptable or certain chromatographic features are unacceptable. The present data show that HbE trait chromatograms are indistinguishable from those with HbAA; fortunately the results from the present study also show that results from these chromatograms are acceptable. Our study data indicate that results for HbD trait are not acceptable for the Variant, results that are consistent with a previous report (12). Although not stated in the instruction manual for the Variant method, HbD trait samples showed chromatograms with “unknown” peaks, one of which separates between the A1c and A0 peaks (Supplemental Data Fig. 1); the careful technician should be able to categorize such chromatograms as unreportable. The instruction manual for the Variant II NU states that specimen results showing HbE and HbD

| Table 1. Mean differences from the comparison method for samples containing either HbE or HbD trait. |
|---------------------------------|-----------------|-----------------|-----------------|
| **Manufacturer** | Method | **HbE trait** | **HbD trait** |
|                  | n | 6% HbA1c | 9% HbA1c | n | 6% HbA1c | 9% HbA1c |
| Immunoassay | | | | | | |
| Bayer/Metrika | A1cNow | 42 | −0.13 | −0.18 | 41 | −0.01 | 0.17 |
| Beckman | Synchron DxC | 42 | −0.06 | −0.16 | 42 | −0.02 | 0.05 |
| Dade Behring | HbA1c on Dimension RxL | 42 | −0.17 | −0.28 | 42 | −0.08 | −0.10 |
| Olympus | HbA1c on Au400 | 42 | 0.01 | 0.22 | 42 | 0.06 | 0.30 |
| Ortho-Clinical | Vitros | 42 | −0.17 | −0.67 | 42 | −0.10 | −0.52 |
| Pointe Scientific | HbA1c on Modular P | 42 | 0.09 | 0.17 | 42 | 0.36 | 0.42 |
| Roche | Integra Gen2 | 42 | −0.24 | −0.40 | 42 | −0.17 | −0.06 |
| Siemens | DCA2000 | 42 | −0.06 | 0.01 | 40 | 0.02 | 0.18 |
| Ion-Exchange HPLC | | | | | | |
| Bio-Rad | D-10 (short) | 42 | 0.40 | 0.41 | 42 | 0.38 | 0.15 |
| Variant II NU | HbA1c Diabetes mode | 42 | −0.24 | −0.47 | 42 | 0.08 | −0.14 |
| Menarini | HbA1c TP mode | 42 | 1.43 | 1.96 | 42 | 1.04 | 1.44 |
| Tosoh | 2.2 Plus | 42 | −1.20 | −2.05 | 42 | 0.24 | 0.03 |
| G7 | 42 | −1.26 | −2.12 | 42 | −0.09 | −0.38 |
| G8 | 42 | −1.41 | −2.42 | 42 | −0.12 | −0.58 |

* Deming regression analysis was performed using the Ultra2 as the comparison method. The mean difference (%) of each of the other methods at clinical decision cutoffs of 6% and 9% were calculated for each Hb trait. To correct for intermethod calibration differences, the mean difference for homozygous HbA samples was subtracted from that calculated for samples containing HbE or HbD trait.
* Methods used are listed in alphabetical order by manufacturer.
* Clinically significant (>0.6% or >0.9% HbA1c at 6% and 9% HbA1c, respectively) differences were found.
* Calculated from only those samples for which an HbA1c result was quantified.

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traits are acceptable and shows typical chromatograms from samples with HbE and HbD trait with both HbE and HbD appearing in the “E,D” window. Our results confirm that results showing HbE and HbD traits are acceptable for the Variant II NU. The instruction manual for the Variant II Turbo does not mention samples with HbE or HbD traits. However, all HbE and HbD trait chromatograms include a peak in the “variant window” that appears on the printed chromatogram as a split A0 peak (Supplemental Data Fig. 2). In the present study, we found these results to be unacceptable for the Variant II Turbo. For the Bio-Rad D-10 short program, the manufacturer makes no claim for HbA1c results with HbE and HbD trait samples. For the short program there is a peak that elutes after the HbA0 peak in the “Variant window” for both HbD and HbE traits. For the D-10 extended program, samples containing HbD have a large “unknown” peak separated after HbA2. HbE appears to be separated in the HbA2 window, causing a very high HbA2 value (up to approximately 30%). We found that results were within clinically acceptable limits (±10% of the HbA1c at 6% and 9%) for both the short and extended program and that results were actually better with the short program.

For the Menarini HA-8160, no statement about HbE or HbD traits is included in the instruction manual. In the diabetes mode, samples containing HbD trait show an extra peak in the “S/C Window” (Supplemental Data Fig. 3). In most cases (75%) the peaks are not labeled, no result is given for HbA1c, and the chromatogram indicates “abnormal separation” (Supplemental Data Fig. 3b). Approximately 25% of HbD trait chromatograms do indicate a HbA1c result and the only distinction is the extra peak in the S/C Window (Supplemental Data Fig. 3c). For those HbD trait samples for which a result is provided, the results are outside clinically acceptable limits. For HbE trait, there is a small extra peak on the trailing edge of the A0 peak that is not completely separated or identified (Supplemental Data Fig. 3d). The present study shows that HbE trait gives unreportable results for the HA-8160 diabetes mode. In the TP mode, HbD trait is indicated by a report of “abnormal separation,” the peaks are not labeled, and no HbA1c result is reported (Supplemental Data Fig. 4). For samples with HbE, the HbE coelutes in the “A2/Var” window, indicating that the A2 result is artificially increased; the present data show that the results for HbAE in the TP Mode are acceptable.

Discussion

Because HbA1c has been shown to be directly related to risk for diabetes complications, the HbA1c test is an essential tool in the management of diabetes. Worldwide clinical diabetes guidelines recommend specific treatment goals related to HbA1c. Previous studies have shown that for some assay methods the presence of Hb variants such as HbS and HbC traits interferes with HbA1c measurement results, but few data were available regarding potential interference from HbD or HbE traits. HbD and HbE traits are 2 of the 4 most common Hb variants both in the US and worldwide. Because patients with these variants usually show no obvious clinical symptoms, physicians may be unaware that their patient with diabetes has one of these variants.

None of the immunoassay, enzymatic, or boron affinity methods we investigated showed clinically significant interference, whereas some of the ion-exchange HPLC methods showed interference from HbE or HbD traits, or both. All of the ion-exchange methods that demonstrated interferences from HbE and/or HbD trait, with the exception of the Variant II Turbo, produced artificially low results. Reporting of such results could lead to undertreatment of hyperglycemia, with concomitant increased risks for complications. In the case of the Variant II Turbo, the artificially increased results seen in the presence of both HbE and HbD traits could result in overly aggressive treatment that could increase risk of hypoglycemia. As mentioned previously, ion-exchange chromatograms must be carefully examined to identify possible interference from these Hb variants so that inaccurate results are not reported. In most (but not all) cases, reporting of inaccurate results can be avoided if manufacturer instructions are followed carefully. For some methods, however, manufacturer instructions alone do not provide sufficient information for making the correct decision about reporting results. Laboratories should be aware of the limitations of their methods with respect to these variants and indicate this information on reports to physicians. Physicians should consider the possibility of interference from an Hb variant if a patient’s HbA1c result is significantly different from what is expected on the basis results of blood glucose self-monitoring and/or other diagnostic test results or clinical symptoms.

In addition to interference of these variant Hbs on the actual measurement method for HbA1c, biological factors that may affect HbA1c concentrations must also be considered. For example, altered erythrocyte lifespan or a variant Hb that glycates at a rate different from that of HbA could result in artificially low or high HbA1c. Reports suggest that erythrocyte lifespan is normal in individuals with HbD trait and homozygous HbE. Given that the amino acid substitutions in HbD and HbE are both far from the primary site of Hb glycation (the N-terminus of the β chain), it seems unlikely that either variant would demonstrate glycation rates that are significantly different from that of HbA.
Nonetheless, further studies are needed to verify this assumption.

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