Published in final edited form as:

Clin Chim Acta. 2016 July 1; 458: 40–43. doi:10.1016/j.cca.2016.04.027.

Higher Degree of Glycation of Hemoglobin S Compared to Hemoglobin A Measured by Mass Spectrometry: Potential Impact on HbA_{1c} Testing

Kuanysh Kabytaev^a, Shawn Connolly^a, Curt L. Rohlfing^a, David B. Sacks^b, Alexander V. Stoyanov^{a,*}, and Randie R. Little^{a,*}

^aDepartment of Pathology and Anatomical Sciences, University of Missouri, Columbia, Missouri, United States

^bDepartment of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland, United States

Abstract

BACKGROUND—Glycated hemoglobin (GHb), reported as HbA_{1c}, is used as marker of long-term glycemia for diabetic patients. HbA_{1c} results from boronate affinity methods are generally considered to be unaffected by most hemoglobin variants; this assumes comparable glycation of variant and non-variant (HbAA) hemoglobins. In this report, glycation of HbA beta chain (βA) and HbS beta chain (βS) for the most common Hb variant trait (HbAS) are examined.

METHODS—We analyzed 41 blood samples from subjects with HbAS, both with and without diabetes. Using LC-MS, ratios of glycated HbS to glycated HbA were determined by comparison of areas under the curves from extracted ion chromatograms.

RESULTS—Glycation of βS chains was significantly higher ($p < 0.001$) than βA chains; this difference was consistent across subjects. Total (α+β) glycated HbAS was theoretically estimated to be ~5% higher than glycated HbAA.

CONCLUSION—This novel mass-spectrometric approach described allows for relative quantification of glycated forms of βS and βA. Although βS glycation was significantly higher than that of βA, the difference in total glycation of HbAS versus HbAA was smaller and unlikely to impact clinical interpretation of boronate affinity HbA_{1c} results. These data support the continued use of boronate affinity to measure HbA_{1c} in patients with HbAS.

Graphical Abstract

*Co-corresponding Authors at: Diabetes Diagnostic Laboratory, Department of Pathology and Anatomical Sciences, University of Missouri, 1 Hospital Drive, Columbia, MO 65212 United States. Tel.: 573-882-1257 (RRL); 573-884-6830 (AS); Fax: 573-884-4748; littler@health.missouri.edu; stoyanova@health.missouri.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Hemoglobin A_{1c}; hemoglobin glycation; mass spectrometry; glycation sites; Hemoglobin S; boronate affinity

1. Introduction

Glycated hemoglobin (GHb) is formed by nonenzymatic glycation of amino groups of hemoglobin. Hemoglobin is constantly subjected to glycation over the life-span of erythrocytes and the proportion of glycated to non-glycated Hb reflects the equilibrium between the rate of this reaction and erythrocyte elimination from the circulation. GHb is therefore used as a marker of long-term (8–12 weeks) glycemic control for diabetic patients [1].

There are a number of different glycation sites on the globin chains of hemoglobin with varying degrees of susceptibility to glycation (different rates of glycation) [2]. Hemoglobin A_{1c} (HbA_{1c}), technically refers to HbA glycated at the N-terminus (Val-1) of the beta chain. However, HbA_{1c} has also become the preferred term for reporting clinical results that are now harmonized to a common reference regardless of the glycated hemoglobin species measured (HbA_{1c}, total GHb) [3].

HbA_{1c} can be measured by several analytical methods, including immunoassay, boronate affinity chromatography, ion exchange chromatography, and electrophoresis. More than 1200 structural hemoglobin mutants have been identified [4] and some variants can produce method-dependent erroneous results with HbA_{1c} analysis that could lead to inappropriate medical treatment [5]. Numerous studies have investigated Hb variant interference on HbA_{1c} values obtained by the most commonly used HbA_{1c} methods [6,7]. Boronate affinity methods separate glycated from non-glycated hemoglobin based on the binding of the *cis*-diol group of the glycated portion of hemoglobin to a phenylboronate gel. In many of these interference studies, boronate affinity HPLC was used as the reference/comparative method because this method should not be affected by changes in the globin chain and is therefore thought to have minimal or no interference from Hb variants. Even though boronate affinity glycated Hb results are harmonized to report HbA_{1c}, all glycated Hb species are actually measured, including glycated variants. A potential source of bias can be a difference in glycation rate for the Hb variant compared to normal HbAA.

HbAS is the most prevalent hemoglobin variant worldwide [4]. A different glycation rate for HbS versus HbA would interfere with boronate affinity results. Our previous study showed that boronate affinity HPLC results for samples with HbAS were comparable to International Federation of Clinical Chemistry (IFCC) Mass Spectrometric Reference Method which had already been shown to have no interference from HbAS [9].

Materials and methods

1.1 Sample preparation

Whole blood samples collected in EDTA-containing tubes from non-diabetic and diabetic subjects with HbAS were obtained from leftover de-identified samples sent for HbA_{1c} analysis. After removing plasma and washing erythrocytes with saline, an erythrocyte hemolysate was made by adding 1.5 ml of DI water and 1.5 ml of CCl₄ to the packed erythrocytes (0.5 ml). The hemolysate was then vortex-mixed and centrifuged at 1000xg for 10 minutes. The upper hemolysate layer was removed and stored at -80°C prior to analysis.

2.2 LC-MS analysis

MS analysis was performed using an API-4000 triple quadrupole mass spectrometer (AB Sciex) coupled to a Shimadzu Prominence LC system and UV detector (Shimadzu Scientific Instruments) integrated with a switching valve (VICI Valco Instruments). Reverse-phase chromatography was performed using a Jupiter 5 μ C18 column (30 mm $\times$ 4.6 mm, 300A, Phenomenex). Two mobile phases were used for elution: (A) 0.4% formic acid in water and (B) 0.4% formic acid in acetonitrile. The total flow rate was 0.5 ml/min. The gradient was: 0–3 min 0% B, 3–10 min 50–100% B, 10–11 min 100–30% B, 11–12 min 30–100% B, 12–13 min, 100–0% B, 13–20 min 0% B. Valco valve positions were follows: 0–3 min into drain (desalting), 3–13 min into ESI-MS, 13–20 min into drain (equilibration). The column and autosampler plate were set at 40°C and 4°C, respectively. The UV detector wavelength was 280 nm. The hemolysate samples (5 μ l) were diluted prior to analysis with 200 μ l of DI water. The volume of injection was 10 μ l. Each sample was analyzed in 4 replicates. The MS parameters were as follows: positive ionization mode, resolution Q1 high, pause between masses 5 ms, time for each mass 50 ms, declustering potential 70V, ion spray voltage 4000 V. For relative quantification, the following masses of intact globin chains were used: 932.1 Da (S β^{17+}), 933.9 (A β^{17+}), 941.6 (S β glycosylated¹⁷⁺), 943.4 (A β glycosylated¹⁷⁺), 945.9 (α^{16+}), 956 (α glycosylated¹⁶⁺). The LC-MS system was operated under Analyst 1.6 software. For each globin chain species (β A, β S, α), the percent glycosylated was determined (glycosylated/glycosylated + non-glycosylated). For more details see appendix A.

2.3 Statistical analysis

Mixed proc in SAS v.9 (SAS Institute Inc.) was used to determine if the mean differences between glycosylated β S and glycosylated β A were significantly different.

3. Results

Fig. 1 shows the mass spectrum of HbS in the 930–960 Da region. In order to estimate the relative amounts of each chain, β S, β A, glycosylated β S, glycosylated β A, α , and glycosylated α , we

developed an LC-MS method based on comparison of the area under the curve of extracted-ion chromatogram (XIC) of charged forms $\beta 17+$ and $\alpha 16+$ globin chains (Fig. 2). These ions were chosen because of their high signal intensity and separation. The imprecision (CVs) for this LC-MS method was $< 2.7\%$, $< 2.5\%$, and $< 2.7\%$ for βS , βA and α glycation, respectively based on the mean CV of each quadruplicate analysis. Fig. 3 shows βS , βA and α glycation for each sample. The means (SDs) for α , βA and βS glycation were 7.39 (2.42), 9.37 (3.28), and 10.91 (3.17), respectively. The mean ratio ($\pm$ SD) of the percentages of glycated βS to glycated βA was 1.19 ± 0.12 ($n=41$); the degree of glycation of each chain is in the same order (α glycated, βA glycated, βS glycated) across all samples. Glycation of HbS was significantly higher ($p<0.001$) than glycation of HbA.

4. Discussion

It is important to report HbA_{1c} results that can be related to clinical studies and to current recommendations for diabetes care and diagnosis. Since some patients with diabetes and those undergoing evaluation for diagnosis of diabetes also have silent heterozygous hemoglobin variants, it is imperative to report accurate HbA_{1c} results in the presence of common hemoglobin variants whenever possible so that clinicians have a good estimate of glycemic status and can make appropriate clinical decisions in these individuals.

Here we observed higher glycation of HbS compared to HbA in subjects with HbAS; the reason for this has not been determined. In order to determine the potential impact of this difference in glycation on clinical interpretation of HbA_{1c} results for subjects with HbAS, it is necessary to consider the glycated fractions that are measured by each HbA_{1c} method. Ion-exchange HPLC methods separate the HbA_{1c} fraction and determine the percentage of HbA_{1c} specifically in relation to the total amount of hemoglobin A while excluding HbS fractions. Thus, the extent of glycation of HbAS does not affect the final HbA_{1c} result measured by HPLC as long as HbAS does not impact the separation of the HbA_{1c} fraction from HbA0 or overlap the HbA_{1c} or HbA peaks. Boronate affinity HPLC measures total glycated hemoglobin (Total GHb) including any glycation on βA , βS and α chains. This total GHb measurement can be expressed as a standardized HbA_{1c} result because of the high correlation between HbA_{1c} and total GHb in subjects with HbAA [10]. Thus, all laboratories can report a standardized HbA_{1c} result regardless of which method type is used.

Because boronate affinity methods are based on binding of the *cis*-diol component of GHb to a boronate affinity resin, and therefore measure all glycated hemoglobins regardless of species, it has been assumed that there is little or no interference from most Hb variants. However, this postulate assumes equivalent glycation of the variant hemoglobin and HbA. In an effort to confirm that boronate affinity HPLC reports correct standardized HbA_{1c} results with HbAS and could be used as a reference/comparative method for evaluation of HbAS interference, boronate affinity results for samples with HbAS were compared to the IFCC Reference Method for HbA_{1c} [9]. The first step in the IFCC procedure is the enzymatic cleavage of hemoglobin at position 6 from the N-terminal end of the β -chain. Since HbS contains a substitution at this position (Glu – Val), there is no enzymatic cleavage of the HbS β chain. Consistent with this, the IFCC method has been shown not to measure N terminal hexapeptide when purified hemoglobin S was analyzed [11]. Therefore, the presence of HbS

trait does not interfere with measurement of HbA_{1c} by the IFCC Reference Method. A prior comparison study showed no statistically significant difference between the boronate affinity/IFCC relationships for HbAS vs. HbAA samples. However, at HbA_{1c} levels 9%, boronate affinity results were slightly higher (0.23% HbA_{1c} at 9% HbA_{1c} based on linear regression) for HbAS versus HbAA samples [9].

If some assumptions are made, the theoretical effect of higher β S glycation, found in the present study, on differences between methods that measure total GHb and HbA_{1c} specifically can be calculated. First, we estimated the ratio of % α glycated to % β glycated for HbA to be 0.64 based on Roberts, et al [12]. The amount of β S in HbAS subjects is generally ~40% [13]. Using the experimentally determined ratio of percentages of glycated β S to glycated β A of 1.19, i.e. β S glycates more than β A, the net increase in total glycation for HbAS compared to HbAA is approximately 5% (see appendix). In other words, there is an approximately 5% increase in the net glycation due to higher glycation of β S compared to β A. The effect of increased glycation of HbS compared to HbA is essentially diluted because there is more HbA than HbS in heterozygous HbAS and glycated α chain is also included in the boronate affinity total GHb. This theoretical estimate is in reasonable agreement with our previous study where boronate affinity results for subjects with HbAS were found to be ~2.5% higher compared to those of HbAA subjects [9]. The slightly lower observed difference compared to the current estimate may be due the inclusion of hemoglobins other than glycated β S, glycated β A and glycated α in the boronate affinity total GHb component, further reducing the relative contribution of glycated β S to the total.

It should be mentioned that we have not addressed the issue of erythrocyte lifespan, we only examined the analytical measurement of Hb glycation. Shortened erythrocyte lifespan results in lowered glycation due to lessened exposure of the erythrocytes to glucose, resulting in artificially lowered HbA_{1c} results regardless of assay methodology [14].

5. Conclusion

We compared the amount of glycation of β A and β S (within-subject) by an LC-MS method. The hemoglobin S β chain was found to be more susceptible to in-vivo glycation compared to that of HbA. Although the difference is statistically significant, it is not large enough to preclude the use of boronate affinity methods to measure HbA_{1c} in patients with HbAS or as a reference for HbAS interference studies. Further studies are needed to determine if the magnitudes of glycation for other common hemoglobin variants such as HbC and HbE are different from that of HbA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the support of NIH/NIDDK. We also acknowledge support from Agilent and the University of Missouri Research Board. We thank Dr. Dmitriy Shin for his help with signal processing and theoretical calculations.

Abbreviations

GHb	glycated hemoglobin
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine

References

1. American Diabetes Association. Standards of Medical Care in Diabetes – 2016. Diabetes Care. 2016; 39(Suppl 1):S40–S43.
2. Wang SH, Wang TF, Wu CH, Chen SH. In-depth comparative characterization of hemoglobin glycation in normal and diabetic bloods by LC-MS/MS. J Am Soc Mass Spec. 2014; 25:758–766.
3. Sacks DB, Arnold M, Bakris GL, et al. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. Diabetes Care. 2011; 34:e61–e99. [PubMed: 21617108]
4. [accessed 02-03-16] A database of human hemoglobin variants and thalassemias. 2016. <http://globin.cse.psu.edu/globin/hbvar/menu.html>
5. Shimizu S, Taira A, Hatazaki M, Umayahara Y, Taira M, Koga M. Two non-diabetic cases of variant hemoglobin treated with oral hypoglycemic agents due to spurious HbA_{1c} elevation. J Japan Diab Soc. 2015; 58:121–127.
6. Lin CN, Emery T, Little RR, et al. Effects of hemoglobin C,D,E, and S traits on measurements of HbA_{1c} by six methods. Clin Chim Acta. 2012; 413:819–821. [PubMed: 22244931]
7. Rohlfing C, Hanson S, Weykamp C, et al. Effects of hemoglobin C, D, E and S traits on measurements of hemoglobin A_{1c} by twelve methods. Clin Chim Acta. 2016; 455:80–83. [PubMed: 26828530]
8. Jaisson S, Leroy N, Desroches C, Tonye-Libyh M, Guillard E, Gillery P. Interference of the most frequent haemoglobin variants on quantification of HbA_{1c}: Comparison between the LC-MS (IFCC reference method) and three routinely used methods. Diab Metab. 2013; 39:363–369.
9. Little RR, Vesper H, Rohlfing CL, Ospina M, Safar-Pour S, Roberts WL. Validation by a mass spectrometric reference method of use of boronate affinity chromatography to measure glycohemoglobin in the presence of hemoglobin S and C traits. Clin Chem. 2005; 51:264–265. [PubMed: 15563476]
10. Little RR, Wiedmeyer HM, England JD, et al. Interlaboratory standardization of measurement of glycohemoglobins. Clin Chem. 1992; 38:2472–2478. [PubMed: 1458587]
11. Jeppsson JO, Kobold U, Barr J, et al. Approved IFCC reference method for the measurement of HbA_{1c} in human blood. Clin Chem Lab Med. 2002; 40:78–89. [PubMed: 11916276]
12. Roberts NB, Green BN, Morris M. Potential of electrospray mass spectrometry for quantifying glycohemoglobin. Clin Chem. 1997; 43:771–778. [PubMed: 9166230]
13. Bain, BJ.; Wild, BJ.; Stephens, AD.; Phelan, LA. Variant Haemoglobins: A Guide to Identification. Wiley-Blackwell; 2010.
14. Cohen RM, Franco RS, Khera PK, Smith EP, Lindsell CJ, Ciralo PJ, Palascak MB, Joiner CH. Red cell life span heterogeneity in hematologically normal people is sufficient to alter HbA_{1c}. Blood. 2008; 112:4284–4291. [PubMed: 18694998]

Highlights

- Percent glycated β A, β S, and α hemoglobins were determined using LC-MS
- LC-MS detected significantly higher glycation of β S chain ($p < 0.001$) versus β A
- For HbS trait, calculations showed 5% higher total ($\alpha + \beta$) glycation versus HbAA
- The data support using boronate affinity to measure HbA_{1c} in HbAS patients

Fig. 1. Mass spectrum of hemoglobin AS in the 930–960 Da region. Specific glycated and non-glycated peaks are indicated.

Fig. 2.
Extracted ion chromatograms of six globin chains: α , α glycosylated, β A, β A glycosylated, β S and β S glycosylated.

Fig. 3.
Magnitude of glycation of α, β, and βS globin chains determined by LC-MS method.