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Higher degree of glycation of hemoglobin S compared to hemoglobin A measured by mass spectrometry: Potential impact on HbA_{1c} testing

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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.

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. >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 [6-8]. 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

Abbreviations: GHb, glycated hemoglobin; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine.

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Method which had already been shown to have no interference from HbAS [9].

2. Materials and methods

2.1. 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 CCl_4 to the packed erythrocytes (0.5 ml). The hemolysate was then vortex-mixed and centrifuged at 1000 ×g for 10 min. 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 × 4.6 mm, 300 A, 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 μl) were diluted prior to analysis with 200 μl of DI water. The volume of injection was 10 μl. 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 70 V, 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 glycated^{17+}), 943.4 (A β glycated^{17+}), 945.9 (α^{16+}), and 956 (α glycated^{16+}). The LC-MS system was operated under Analyst 1.6 software. For each globin chain species (βA, βS, α), the percent glycated was determined (glycated/glycated + non-glycated). 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 glycated βS and glycated βA 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, βS, βA, glycated βS, glycated βA, α, and glycated α, we developed an LC-MS method based on comparison of the area under the curve of extracted-ion chromatogram (XIC) of charged forms β17+ and α16+ 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 (±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

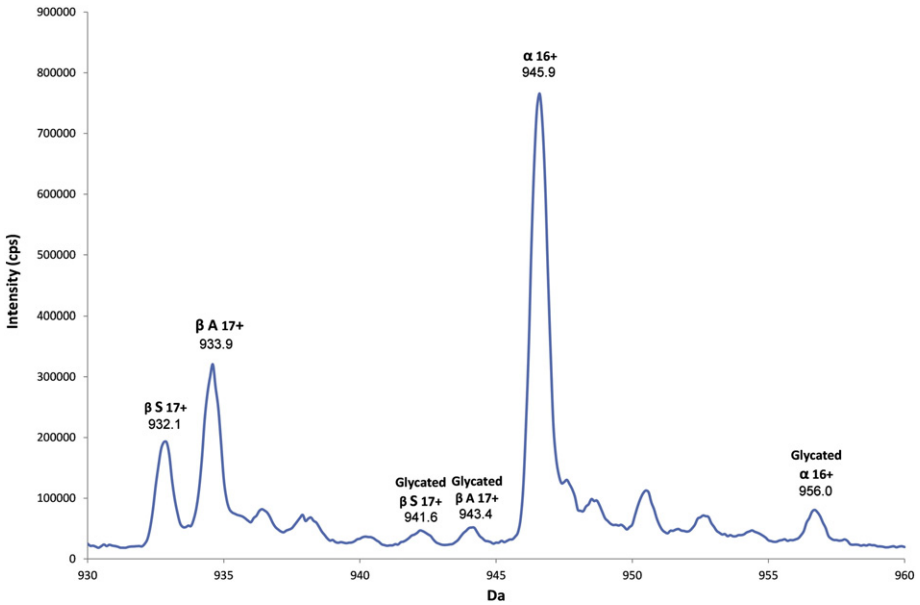

Fig. 1. Mass spectrum of hemoglobin AS in the 930–960 Da region. Specific glycated and non-glycated peaks are indicated.