Rapid Publication

Glycosylated Hemoglobins: Increased Glycosylation of Hemoglobin A in Diabetic Patients

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SUMMARY

The components of the hemoglobin-A_1 fraction —hemoglobins A_{1a-c}—arise from nonenzymatic glycosylation of hemoglobin A at the β-chain N-terminal amino groups and can be resolved from hemoglobin A by cation exchange chromatography. Glycosylation can also occur at the α-chain N-terminals as well as the ϵ-amino groups of lysine residues of both α- and β-chains; this results in glycosylated species appearing in the hemoglobin-A fraction. In this study, we determined the extent of hemoglobin-A glycosylation using a colorimetric chemical method specific for the detection of ketoamine-linked hexoses in proteins. We demonstrate increased glycosylation of the main hemoglobin-A fraction in diabetic patients, which correlates significantly ($r = 0.72$, $P < 0.001$) with the hemoglobin-A_1 percentage determined by column chromatography in the corresponding hemolysates. This finding provides the basis for the application of this chemical procedure to the measurement of total glycosylation of hemoglobin. DIABETES 28:337–340, April 1979.

Cation exchange chromatography of hemolysates of red blood cells resolves four minor hemoglobin (Hb) components from the main Hb-A fraction. These minor components—Hb-A_{1a1}, Hb-A_{1a2}, Hb-A_{1b}, and Hb-A_{1c}—collectively referred to as the Hb-A_1 fraction, comprise about 7% of the total hemoglobin in normal subjects and result from post-translational, nonenzymatic modification of Hb-A.[1] Hb-A_{1c} is the most abundant of the minor components and is present in increased amounts in patients with diabetes mellitus as a consequence of increased blood glucose levels. In Hb-A_{1c}, glucose is attached to the N-terminal amino group of the β-chains by a stable ketoamine linkage.[2] Hb-A_{1a1} and Hb-A_{1a2} are the β-chain N-terminal adducts of fructose-1,6-diphosphate and glucose-6-phosphate, respectively,[3] while Hb-A_{1b} is thought to result from a deamidation in the β-chain of Hb-A_{1c}.[4] The recent demonstration that glucose is attached by a ketoamine linkage to the ϵ-amino groups of lysine residues in both α- and β-chains[5] indicates that glycosylation of the molecule is not unique to the N-terminal positions.

Flückiger and Winterhalter[6] applied a colorimetric test, which appears to be specific for the ketoamine-linked hexoses, to the determination of Hb-A_{1c}. In this procedure, furfural compounds generated from the carbohydrate moieties on heating under acidic conditions are quantitated colorimetrically with 2-thiobarbituric acid (TBA). Using a modification of this method, we were able to detect increased glycosylation of the Hb-A fraction from patients with diabetes mellitus. This increase in glycosylation of Hb A at sites other than the N-terminal amino groups is shown to correlate with the increase in Hb-A_1 in these hemolysates. These findings indicate that glycosylation of the hemoglobin molecule at the various reactive sites increases progressively with increasing hyperglycemia and that chemical measurement of total glycosylation of hemoglobin (Hb-A_1 and Hb-A) provides a useful alternative to the currently used chromatographic procedures for the determination of glycosylated hemoglobins.

MATERIALS AND METHODS

Blood specimens from insulin-dependent diabetic and normal nondiabetic subjects were collected in EDTA tubes. Preparation of red blood cell hemolysates and quantitation of the Hb-A_1 fraction by column chromatography on Biorex-70 were performed as previously described.[7] More recently, the reproducibility of the method was improved by maintenance of the columns at 28°C in a water bath, since a 1°C change in temperature significantly affects Hb-A_1 quantitation. The columns were considered properly equilibrated only when the pH of the effluent was identical to that of the starting buffer, i.e., 6.76 ± 0.01. In addition, two pools

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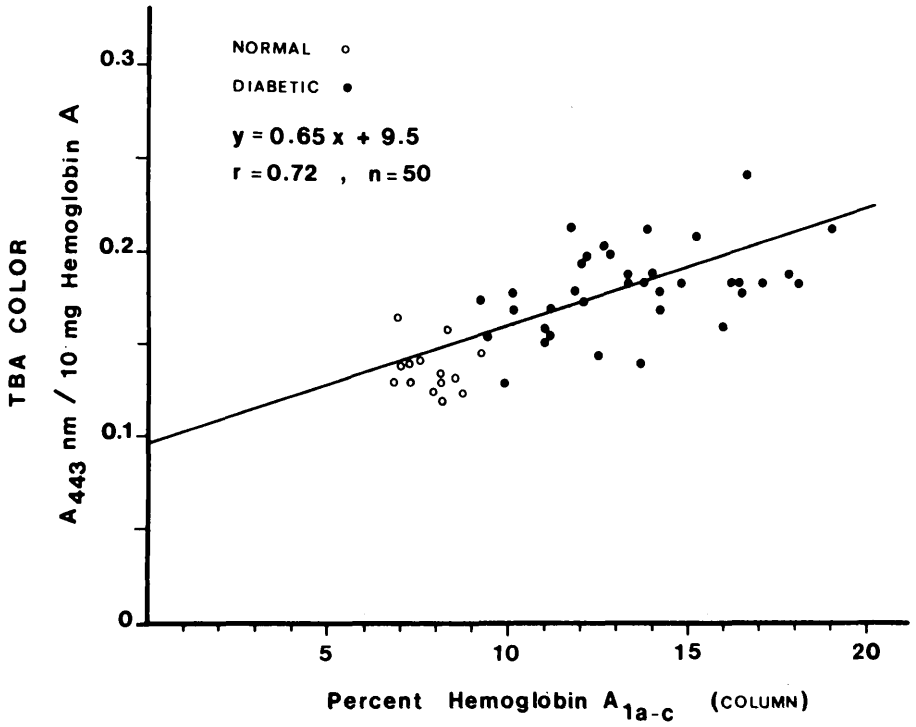


FIGURE 1. Correlation of the increase in TBA color value of the Hb-A fraction to the Hb-A_1 percentage levels of the respective hemolysates.

of hemolysates, normal and diabetic, frozen in liquid nitrogen and stored in aliquots at −90°C, were run with each batch of samples for longitudinal quality control.

The Hb-A fractions isolated from normal and diabetic hemolysates during the quantitation of Hb-A_1 content by column chromatography were assayed by the colorimetric thiobarbituric acid procedure (TBA test) as follows: a 0.25 vol of cold 40% trichloroacetic acid (w/v) was added to a 10-mg aliquot of the Hb-A fraction, and the mixture was centrifuged at 2500 rpm for 15 min. The supernatant was discarded, and the precipitate was suspended in 1 ml of distilled water and further processed by the TBA test. Hemoglobin concentrations were determined by the method of Drabkin.[8]

Colorimetric test. The TBA test[6] was modified to increase color yield and enhance reproducibility by increasing the oxalic acid concentration and the duration of heating of the mixture. The amount of hemoglobin was 9 to 11 mg per test, which is essential for attainment of reproducible results. The modified method is as follows: samples of hemolysates containing about 10 mg hemoglobin were diluted to a volume of 1 ml with distilled water. After the addition of 0.5 ml of 1.0 N oxalic acid, the tubes were mixed and capped with rubber stoppers vented with 26-gauge needles. The tubes were placed in a heating block at 100°C, and the needles were removed after a 10-min equilibration period. Heating was continued for a total of five hours. The tubes were removed and allowed to cool in an ice bath. Cold 40% trichloroacetic acid (0.5 ml) was added to each tube, and the contents were mixed and centrifuged. A 1.5-ml aliquot of the supernatant was pipetted off and 0.5 ml of a 0.05-M aqueous 2-thiobarbituric acid solution was added with mixing. After a 15-min incubation at 37°C, the tubes were allowed to stand at room temperature for 20 min before reading of the samples at 443 nm in a Beckman spectrophotometer. The efficiency of 5-hydroxymethylfurfural (5-HMF) liberation from Hb-A_{1c} was estimated by comparison with the amount of adduct formed between TBA and known amounts of 5-HMF. The millimolar extinction coefficient of the adduct was 26 at 443 nm.

Standards for the TBA test were prepared from Hb-A_{1c} and Hb-A purified from the same hemolysate pool on a preparative Biorex-70 column, 8 × 100 cm, as described by McDonald et al.[3] These standard solutions varied in Hb-A_{1c} content from 5 to 15% and were lyophilized in 10-mg aliquots for long-term use. In addition, 10-mg aliquots of the same Hb-A fraction were also lyophilized and run as blanks with each standard curve.

RESULTS

Figure 1 shows the TBA color values determined on aliquots of Hb-A isolated during the column chromatographic quantitation of Hb-A_1. The TBA color values are increased in the Hb-A obtained from diabetic hemolysates, and they correlate significantly ($r = 0.72$, $P < 0.001$) with the Hb-A_1 levels of the respective hemolysates. This finding indicates increased glycosylation of the Hb-A fraction in the diabetic state as a function of hyperglycemia.

The variable TBA color values of Hb-A in different blood samples precluded the use of a simple standard curve for a direct reading of Hb-A_1 percentages by the TBA method. However, the correlation of this increase in glycosylation of Hb-A at sites other than the N-terminal positions (i.e. ϵ-amino groups of lysine residues) with the chromatographically determined Hb-A_1 percentage permits direct estimation of total glycosylation in hemolysates. Figure 2 shows that the TBA color values determined directly on total hemolysate hemoglobin correlate significantly and linearly ($r = 0.89$, $P < 0.001$) with the Hb-A_1 percentage determined by column chromatography. The correlation between the two methods did not hold in hemolysates of patients with hemoglobins that have altered chromatographic mobilities, such as Hb-C and -S, and persistent Hb-F.

Figure 3 shows the mean ± SD of 10 standard curves

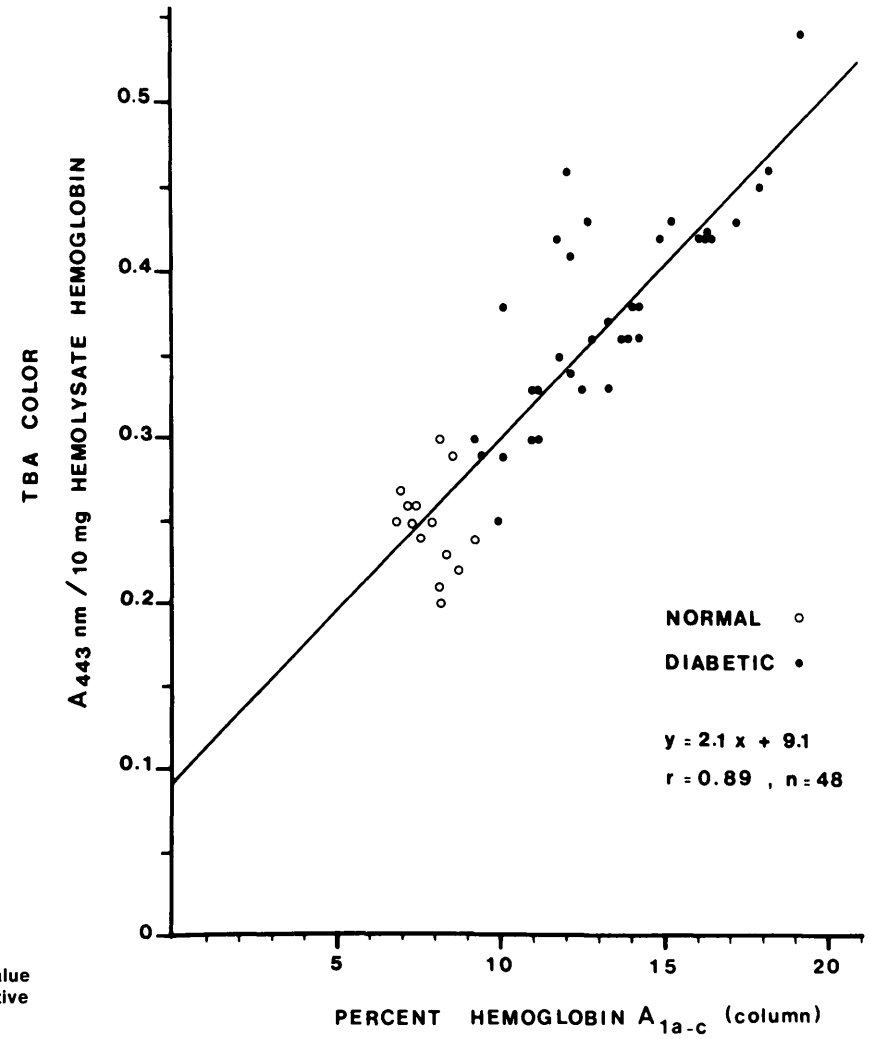


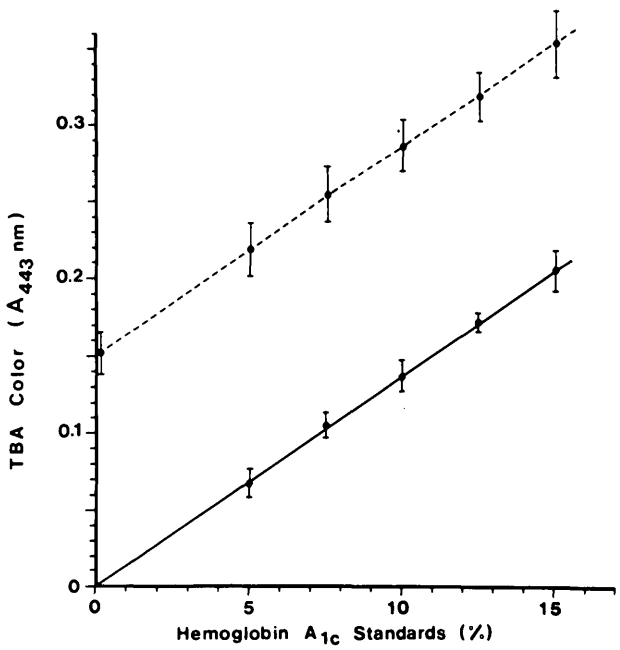
FIGURE 2. Correlation of increase in TBA color value of normal and diabetic hemolysates to the respective Hb-A_1 percentage levels.

obtained over a 16-mo period with the lyophilized 10-mg samples of known Hb-A_{1c} content. The TBA color values increased linearly with increasing Hb-A_{1c} content. The Hb-A blank value in this batch of standards was 0.151 ± 0.014 OD $_{443}$/10 mg Hb-A. Subtraction of the TBA color value of the Hb-A blank produces standard curves with the intercept at the origin. Based on the specific color yield of Hb-A_{1c}, a 35% efficiency of 5-HMF formation is calculated. The calculation assumes the presence of 1 mol of glucose per mole $\alpha\beta$ dimer at the β N-terminal position in Hb-A_{1c} and is based on the experimentally determined millimolar extinction coefficient of 26 (OD $_{443}$) for the 5-HMF adduct. Assuming a similar 35% efficiency for the detection of glucose in Hb-A, we calculate that 0.11 mol of glucose is present per mole of $\alpha\beta$ dimer in this Hb-A fraction.

DISCUSSION

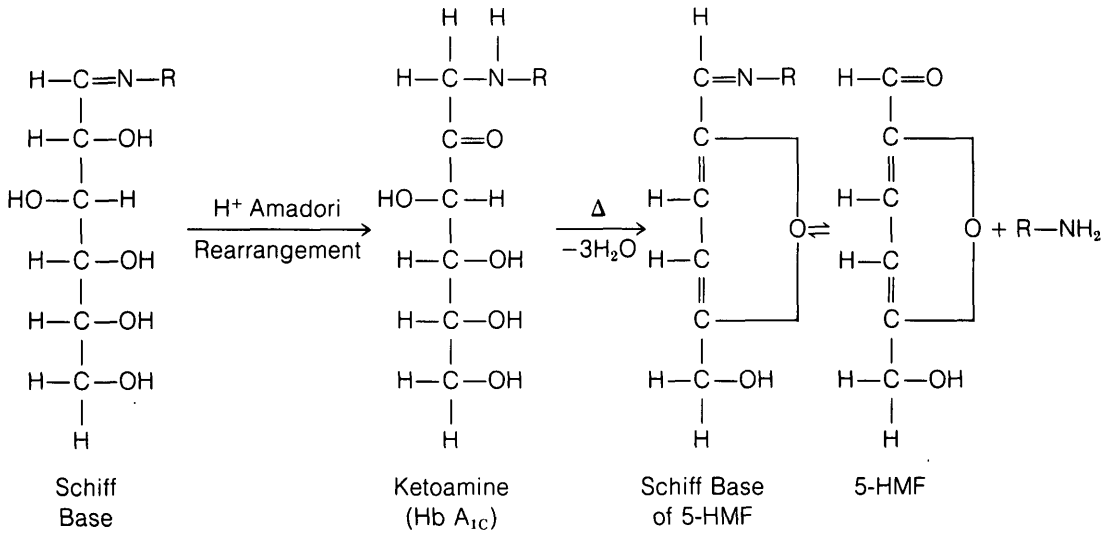
Previous studies have shown that, in Hb-A_{1c}, a glucose molecule is attached to the N-terminal amino group of each β-chain as a consequence of a nonenzymatic post-translational modification reaction.[1] Nonenzymatic glycosylation is not unique to the β N-terminal, and it was recently demonstrated[5] that both the α N-terminal and the ϵ-amino groups of the lysine residues in both α- and β-chains of Hb-A can also be glycosylated. At all three sites, the glycosylation reaction requires initial Schiff base formation between glu-

FIGURE 3. Reproducibility of the TBA test procedure. Shown are the means ± 1 SD of 10 standard curves with lyophilized hemoglobin samples of known Hb-A_{1c} content. The absolute A_{443} values are denoted by the broken line, and the specific Hb A_{1c} values, after subtraction of the Hb A blank value, are denoted by the solid line.



cose and the amino group and subsequent stabilization in the ketoamine form via the Amadori rearrangement.

The TBA reaction is specific for the detection of ketoamine-linked carbohydrate residues in proteins:



In the presence of oxalic acid, which promotes the Amadori rearrangement, the equilibrium is driven to the ketoamine form, which, after heating, dehydrates and results in the liberation of 5-HMF. The latter can be detected spectrophotometrically as an adduct of 2-thiobarbituric acid.

Our data show that glycosylation of Hb-A at sites other than the β N-terminal increases in proportion to the increase in Hb-A_1 in the diabetic state. This finding demonstrates that hemoglobin glycosylation is general and nonspecific, with its extent determined primarily by the prevailing glucose concentration and the relative reactivity of the various amino groups. The presence of 0.11 mol glucose per mole of $\alpha\beta$ dimer of Hb-A confirms previous estimates derived from studies using tritiated borohydride reduction[9] and C^{14}-glucose incorporation in vitro into Hb-A.[5] Since an $\alpha\beta$ dimer of Hb-A contains 22 lysine residues, we calculate that 0.5% of all lysine residues are glycosylated in the total normal Hb-A peak. This figure is an overestimate of ϵ-amino group glycosylation, since the leading edge of the Hb-A peak is enriched in the Hb-A glycosylated at the α N-terminus.[5] The data thus suggest that the β N-terminal is at least 10 times more readily glycosylated than the ϵ-amino groups (0.5% of total ϵ-amino groups vs. 5% of the β N-terminals).

The reactivity of these amino groups reflects the differences in their pKa values. For instance, in the deoxy state the β N-terminal amino groups have a pKa value of 6.8, while the α N-terminal groups have a pKa of 7.8 and are, additionally, in defined salt bridges. These pKa differences lead to 30% more adduct formation when deoxyhemoglobin is incubated with glucose as compared with incubations with liganded hemoglobin.[6,10] Since the ϵ-amino groups of lysine residues generally have pKa values of about 10, it would be predicted that these groups become glycosylated to a lesser degree.

These data show that chemical measurement of total glycosylation of hemoglobin provides an independent and potentially more useful approach than do current chromatographic methods for Hb-A_1 quantitation. The stability of the lyophilized glycosylated hemoglobin standards in this procedure permits interlaboratory and intralaboratory quality control, which is essential for long-term studies. Finally, the method is generally applicable to measurement of total hemoglobin glycosylation, where the conditions for chromatographic separation of the glycosylated components are not defined.

ACKNOWLEDGMENTS

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