Quantitation of Glycosylated Hemoglobin

Elimination of Labile Glycohemoglobin During Sample Hemolysis at pH 5

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SUMMARY

A simple method for the elimination of labile glycohemoglobin in the chromatographic quantitation of glycosylated hemoglobin is described. Use is made of the instability of Schiff base adducts in acidic solution. Erythrocytes are lysed with a pH 5 buffer. At this pH dissociation reaches completion during sample preparation. **DIABETES *31*:630–633, July 1982.**

Measurement of glycosylated hemoglobin has gained widespread use because its levels reflect averaged long-term blood glucose concentrations.[1,2] Its quantitation by chromatographic means relies on the change of charge of the hemoglobin molecule caused by glycosylation of the amino-termini of the beta-chains. The beta-N-terminally glycosylated hemoglobins elute early from a cation-exchange resin and are designated in order of their elution as HbA_{1a-c},* collectively referred to as HbA_1 or "fast hemoglobin.[11] Glycohemoglobin in which the carbohydrate moieties —glucose in HbA_{1c}—are attached by a Schiff base or aldimine linkage (labile HbA_1) has a charge similar to glycohemoglobin where the linkage has rearranged to the stable ketoamine (stable HbA_1). As a consequence, the two forms are not resolved by most chromatographic techniques.

Aldimine adducts are formed rapidly and reversibly while the stable ketoamine accumulates slowly throughout the 120-day life span of the erythrocyte.[3,4] Measurement of variable proportions of the rapidly formed glycohemoglobin together with the stable glycohemoglobin has caused uncertainty as to the value of glycohemoglobin as an index of long-term control of hyperglycemia.[5,6]

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* Unless specified, HbA_{1c} refers to the composite fraction containing labile and stable HbA_{1c}.

Various procedures have been used for the elimination of labile glycohemoglobin in the quantitation of HbA_1, including incubation of erythrocytes with normal saline,[7] dialysis or ultrafiltration of hemolysates,[6,8] and incubation of erythrocytes with semicarbazide/aniline at pH 5.[9]

We have studied the stability of labile glycohemoglobin at different pH values using high-pressure liquid chromatography (HPLC) for quantitation of HbA_{1a+b} and HbA_{1c}. It was found that labile glycohemoglobin dissociates rapidly and quantitatively if hemolysis is allowed to proceed at pH 5 and 37°C.

MATERIALS AND METHODS

Chemicals. Bio Rex-70 cation-exchange resin, minus 400 mesh, was purchased from Bio-Rad, Inc. (Richmond, California) and glucose oxidase type V from *Aspergillus niger* (E.C. No. 1.1.3.4.) from Sigma Chemical Company (St. Louis, Missouri). All other reagents were reagent grade and obtained from Merck (Darmstadt, BRD).

Apparatus. The HPLC system consisted of a Rheodyne model RE 71-25 injection valve with a 10-μl sample loop, an Altex 3-way manual rotary valve, an Altex 100A solvent metering pump, a Bioanalytical Systems column heater, a Uvicon LCD 725 spectrophotometer (Kontron AG, Zürich, Switzerland), and a Chromatopac C-R1A data processor (Shimadazu Corp., Kyoto). The stainless steel column (4.5 × 125 mm) was adapted by our technical service.

Chromatography. The HPLC method was similar to those previously described.[10–12] The column was equilibrated with a low-phosphate buffer (pH 6.68, 0.005 M $NaH_2PO_4 \cdot H_2O$, 0.014 M Na_2HPO_4, and 0.015 M KCN), maintained at 28°C, and operated at a pressure of 80 psi with a flow rate of 0.69 ml/min. The effluent was monitored at 416 nm and the peak areas integrated. Hemolysate (10 μl) containing 10 μg of hemoglobin was injected and HbA_1 components eluted with a high-phosphate buffer (pH 6.4, 0.104 M $NaH_2PO_4 \cdot H_2O$, 0.049 M Na_2HPO_4) for 20 min. The equilibration time between runs was 7 min. The resulting chromatogram is shown in Figure 1.

Subjects and sample preparation. Blood was obtained

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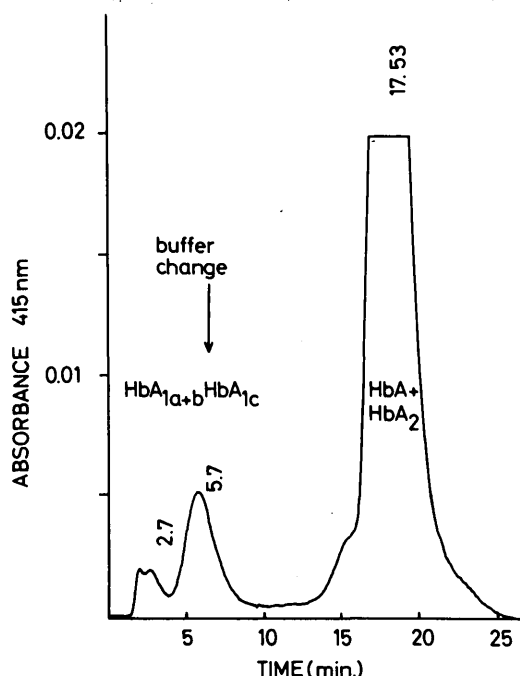


FIGURE 1. Typical chromatogram of a hemolysate resolved by HPLC showing resolution, designation, and retention times of the minor hemoglobin components.

from 10 normal subjects and 40 diabetic patients. The diabetic patients were outpatients cared for at the Kantonsspital Basel and comprised both type I and II diabetics not suffering from acute illness or advanced renal failure. Blood samples were collected in EDTA tubes. Erythrocytes were separated from plasma by centrifugation (1000 × *g* for 10 min) and washed three times with 0.9% saline. For the determination of total HbA_1 erythrocytes were lysed by the addition of 5 vol cold distilled water, and for the determination of stable HbA_1 by the addition of 50 vol 0.05 M potassium biphthalate lysing buffer, pH 5, and incubation at 37°C for 15 min after mixing. The ghosts were removed by centrifugation (2000 × *g* for 10 min). The hemolysate was diluted to 1 mg Hb/ml with sample diluent (0.06 M Na-phosphate, pH 6.6) and passed through a 0.2-μm filter (MF 1, Bioanalytical Systems, Inc., Lafayette, Indiana).

Formation and dissociation of labile glycohemoglobin. Hemolysates rich in labile glycohemoglobin were obtained by incubation of packed erythrocytes with 4 vol of phosphate-buffered saline containing 55.5 mmol/L glucose for 4 h at 37°C. The labile glycohemoglobin was dissociated by hemolyzing erythrocytes with the pH 5 buffer as above. Alternatively, erythrocytes were incubated with (1) 5 vol 0.9% saline for 12 h at 37°C, (2) glucose oxidase (150 U/ml in 0.05 M PBS, pH 6), or (3) 10 vol 30 mM semicarbazide in 84.5 mM sodium acetate-buffered saline, pH 5, at 37°C. The incubated erythrocytes were lysed with water and diluted with sample diluent for injection.

Data analysis. The first-order off-rate constants were estimated based on the three parametric single exponential function $[y(t) = A_0 + A_1 \times \mathrm{Exp}(-A_2t)]$ by a weighted nonlinear least-square curve-fitting procedure. The BASIC program as described in ref. 13 was adapted on an APPLE II computer. Least-squares regression was used for line fitting.

RESULTS AND DISCUSSION

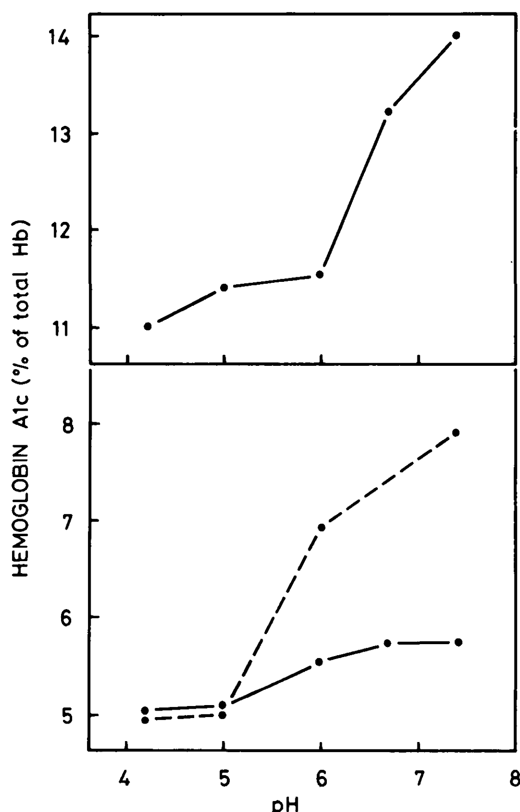
The stability of labile HbA_{1c} at different pH values is shown in Figure 2. Blood specimens were incubated at the respective pH, and total HbA_{1c} was determined immediately. While at pH 7.4 labile HbA_{1c} accounts for 10–20% of total HbA_{1c}, no labile HbA_{1c} is detectable at and below pH 5. Labile HbA_{1c} formed by incubation of erythrocytes with high concentrations of glucose behaves similarly. This pH-dependent stability is characteristic for glycosylamines in general[14] and is in agreement with earlier observations concerning the formation of HbA_{1c}.[15]

The kinetics of dissociation of labile HbA_{1c} at pH 5 and 6 are compared in Figure 3. At pH 5 dissociation reaches completion within 15 min. With increasing pH, decomposition slows down markedly. At pH 6, 35 min is required for total decomposition and at pH 7.4 12 h.

At pH 5 the off-rate constant is 0.48 ± 0.13 min^{-1}. Inclusion of semicarbazide to remove free glucose does not accelerate the dissociation significantly. The respective off-rate is 0.35 ± 0.05 min^{-1} and within the experimental error identical to that of the incubation without semicarbazide. Also, inclusion of aniline, which has been used as a catalyst of the reaction, was without effect.

The rate of dissociation at pH 6 is 0.10 ± 0.04 min^{-1} and is also not increased by removal of free glucose from the equilibrium. Incubation with glucose oxidase yielded an off-rate constant of 0.13 ± 0.01 min^{-1} at pH 6. Incubation of erythrocytes at pH 7 with glucose oxidase accelerated the disappearance of labile HbA_{1c} markedly (data not shown)

FIGURE 2. Stability of labile HbA_{1c} at different pH values. Blood sample from diabetic (upper panel) and normoglycemic (lower panel) individual. (• --- •) Same specimen incubated with glucose. The buffers used were pH 4.2–6.0, 0.05 M K-biphthalate; pH 6.5–7.4, 0.06 M Na-phosphate.



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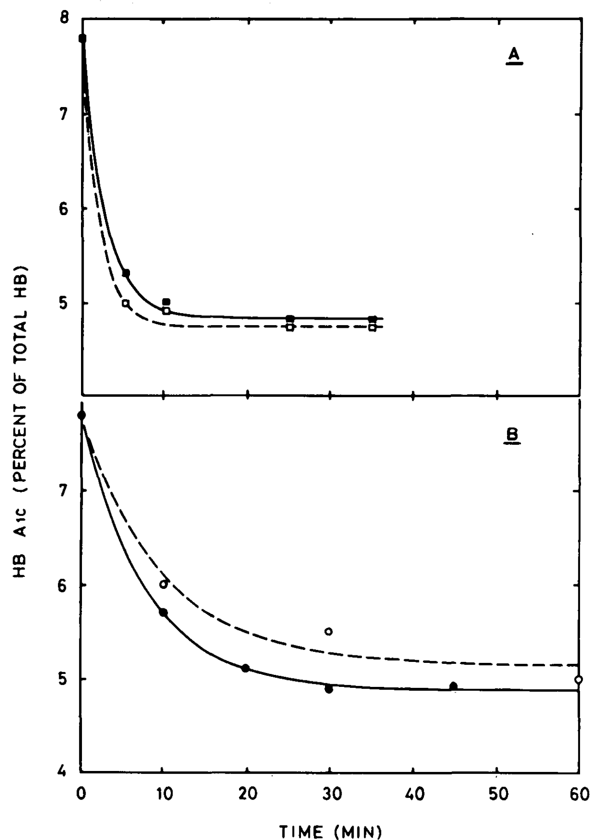

FIGURE 3. Kinetics of dissociation of labile HbA_{1c}. Incubation A: at pH 5 with (■—■) and without (□---□) semicarbazide. B: at pH 6 with (●—●) and without (○---○) glucose oxidase. The curves represent the computer-fitted values for the off-kinetics.

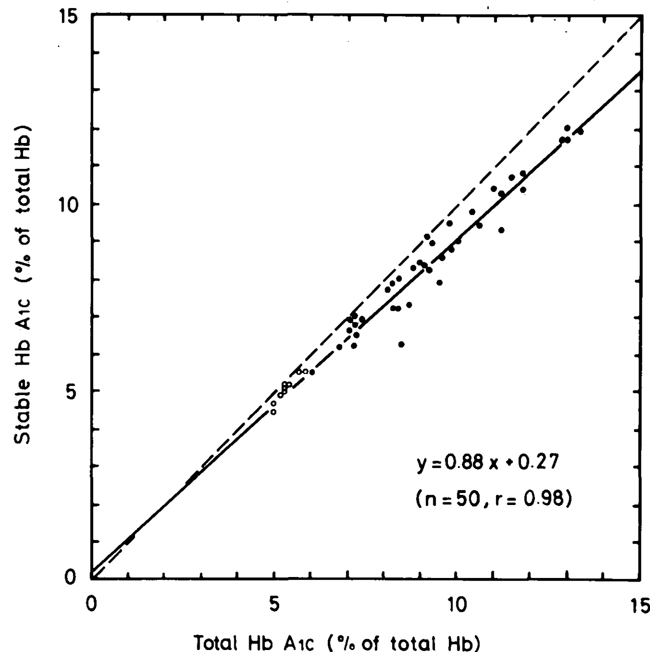

FIGURE 4. Relationship between total (labile and stable) and stable HbA_{1c} in 10 normoglycemic (○) and 40 hyperglycemic (●) individuals. The dotted line represents identity while the solid line is derived from the experimental values.

but the rate was still slower than at pH 6. At pH 7 the kinetics were complex and a rate constant could not be determined.

These data show that the hydrolysis of labile HbA_{1c} is acid catalyzed, as expected for a Schiff base, and that removal of free glucose has no effect on the rate of decomposition under acidic conditions.

Labile HbA_{1c} was therefore eliminated by simply exposing the hemoglobin to pH 5 for 15 min during the hemolysis step. Labile HbA_{1c} was determined by these means in 10 normoglycemic and 40 hyperglycemic individuals. Labile HbA_{1c} accounts for 0.28 ± 0.11% (SD) of total hemoglobin (range 0.1–0.5%) in the normoglycemic range. In the hyperglycemic subjects its concentration was 0.80 ± 0.49% (SD) and more variable (range 0.1–1.2%). This amount of labile HbA_{1c} leads to an overestimation of the long-term blood glucose concentration on the average of 30 mg/dl and in extreme situations 40 mg/dl (calculation based on data in ref. 16).

The relationship between total and stable (i.e., total − labile) HbA_{1c} is shown in Figure 4. Labile HbA_{1c} in the entire sample averages approximately 12% of total HbA_{1c}. This value agrees well with values obtained by other methods for the removal of labile glycohemoglobin.[7,17,18]

The increase in HbA_{1c} upon incubation with glucose and the decrease obtained by various elimination procedures is accompanied by similar but proportionally smaller changes in HbA_{1a+b}. The pH 5 hemolysis step decreases the percentage of HbA_{1a+b} by 0.1% (range 0–0.02%) in normoglycemic and by 0.3% (range 0–1.2%) in hyperglycemic individuals. It is therefore unlikely that the changes in HbA_{1a+b} are incubation artifacts. They are also not caused by inadequate integration, because the values did not change if resolution between HbA_{1a+b} and HbA_{1c} was increased by decreasing the flow rate. It is not possible from our experiments to identify the component(s) in the HbA_{1a+b} fraction responsible for these fluctuations.

Under the conditions used, exposure of hemoglobin to pH 5 does not alter other relevant properties of the molecule. No spectral changes could be detected nor were the chromatographic properties altered. If stored at 4°C, the dilute hemolysates remained stable for 2 wk at pH 5.

A pH 5 step for the removal of labile glycohemoglobin has been previously utilized.[9,19] In one report, labile glycohemoglobin produced by glucose incubation was removed by exposure to pH 5 for 1 h. The utility of this step as an analytic tool was not evaluated, however. In the procedure for the removal of labile glycohemoglobin proposed by Nathan et al.,[9] erythrocytes are incubated with a semicarbazide/aniline reagent. We found that these additions have no significant effect on the rate of dissociation (Figure 2A). We have noted the aniline-containing reagent to turn brownish during incubation, thereby obscuring visible detection of hemolysis. At pH 5 hemolysis is evident after 40 min incubation. Direct hemolysis at pH 5 avoids possible underestimation of HbA_{1c} caused by preferential lysis of the older erythrocytes that are rich in glycohemoglobin.

The simple method proposed should allow elimination of labile glycohemoglobin in all chromatographic techniques and render results more comparable. It also makes possible the estimation of labile and stable glycohemoglobin with little additional effort.

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