Hemoglobin A₁c by Isoelectric Focusing

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We separated the hemoglobins in an unwashed erythrocyte hemolysate according to their isoelectric points on a thin-layer horizontal polyacrylamide gel containing ampholyte (40 g/L) over a pH gradient of 6-8. We scanned the fixed, unstained gels by microdensitometry and calculated the percentage of hemoglobin A₁c. The overall CV (between-run imprecision) for a normal hemolysate (mean, 5.2% of total hemoglobin) stored at -70 °C was 12.6%. An above-normal pooled specimen (mean 8.5%) showed an overall CV of 8.4%. We confirmed that hemoglobin took various degrees of control were 7.6% in the group rated "good," 9.9% in the "fair" group, and 12.2% in the "poor" group. Results for patients' samples (γ) were compared with results by cation-exchange chromatography (δ). The slope was 1.0 and the intercept was 2.2%. The percentage of hemoglobin A₁c in erythrocytes remains constant for seven days in samples stored at 30 °C.

Additional Keyphrases: reference interval • practicality of isoelectric focusing in the routine laboratory • results for diabetics • glycohemoglobin

Interest in the measurement of hemoglobin A₁c (HbA₁c) has escalated with the increasing recognition that the measurement may be useful in the diagnosis and management of chronic hyperglycemia, especially in diabetes mellitus (1-3). Methods of analysis for HbA₁c include colorimetry (4), electrophoresis (5), and isoelectric focusing (6), but the most popular methods are based on cation-exchange chromatography (7, 8). These column methods present several difficulties when used in the routine clinical laboratory. (a) Meticulous control of method variables is required for proper performance. For example, the pH of the eluting buffer should not vary by more than ±0.001 pH unit (7). (b) All methods depend upon a single supplier of the resin. (c) Components other than HbA₁c appearing in the fast chromatographic fraction (HbA; glycohemoglobin) measured in most methods have not been fully defined or characterized (9). In addition, hemoglobin degradation products elute in the fast fraction and may cause artificial increases in HbA₁c in stored samples.

The greater specificity of HbA₁c by isoelectric focusing makes it an attractive alternative to chromatography, but some observers have commented that "the separation is not, however, adequate for quantitation or isolation of HbA₁c" (10) and that "the technical difficulties involved in this method do not permit its routine clinical application" (11). We describe an accurate and precise assay for HbA₁c by isoelectric focusing for use in the routine clinical laboratory.

Materials and Methods

Apparatus

We used an LKB Multiphor System and a High Voltage Power Supply from LKB, Bromma, Sweden, with a circulating cooling water bath from Blue M Electric Company, Blue Island, IL 60406. For the densitometry, we used a Model 4510 densitometer from E. G. and G. Ortec, Oak Ridge, TN 37830, with a 546.1-nm visible-bandpass filter from Ditric Optics, Inc., Marlboro, MA 01752. We obtained 12.5 × 17.8 cm glass plates from the Van Nuyss Glass Co., Van Nuyss, CA 91405, and we cut a gasket for molding the gel from silicone rubber (0.8 mm thick) from McMaster-Carr Co., Santa Fe Springs, CA 90670.

Reagents

All reagents were obtained from Mallinckrodt Inc., St. Louis, MO 63147, except as specified: N,N'-Methylenediisacrylamide, N,N,N',N'-tetramethylisocyanate, and ammonium persulfate from Bio-Rad Laboratories, Richmond, CA 94804.

Ampholine, pH 6-8 ampholytes, from LKB.

Beta-alanine, from Calbiochem, La Jolla, CA 92037.

Potassium cyanide solution, 0.5 g/L of distilled water.

Phosphoric acid, 0.1 mol/L. Dilute 6.6 mL of 15 mol/L (concentrated) H₃PO₄ to 1 L with distilled water.

Trichloroacetic acid, 125 g/L of distilled water.

Sodium hydroxide, 0.1 mol/L.

Donor Selection

We used data from blood samples from 76 non-diabetic adults in calculating a reference interval (12). We also obtained blood from 29 diabetic patients attending a local outpatient clinic. Thirteen had been rated to be in "good" control, nine in "fair" control, and seven in "poor" control according to described criteria (13).

Procedure

Preparation of gels. Assemble the molds, consisting of silicone rubber gaskets at the edges of two glass plates clamped with binder clips, leaving a small space in the gasket to pour in the acrylamide liquid. Dissolve 6.79 g of acrylamide1 and 0.21 g of N,N'-methylenediacrylamide in 90 mL of distilled water and add 10 mL of ampholyte solution (4 g per 10 mL of distilled water) and 4 g of β-alanine. Degas 25 mL of the mixture for 10 min and add 5 μL of N,N,N',N'-tetramethylenediamine, followed by 100 μL of freshly prepared 50 g/L ammonium persulfate solution. After mixing and degassing for one extra minute, transfer this solution to the molds, being careful to avoid air-bubble formation. Close the mold and leave to polymerize at 4 °C for 1–2 h.

Routine procedure. Hemolyze 0.5 mL of unwashed erythrocytes by shaking the cells vigorously in a test tube with 1 mL

1 Liquid or solid polyacrylamide must be handled with neoprene gloves and with use of dust and mist respirator mask, in an operating fume hood. After gel formation, gloves are sufficient.
of distilled water and 1.5 mL of carbon tetrachloride. After centrifugation (15 min, 1000 × g) remove the clear red hemolsate (upper phase). It can be stored at −70 °C until further diluted with two volumes of potassium cyanide solution or to a concentration that will show an absorbance of 70% full scale in the hemoglobin A₀ band on the densitometer. Allow the gel in the mold to reach room temperature. Remove the covering glass and rubber gasket and allow any remaining liquid to drain onto absorbent paper. Place the plate on the bed support and attach a submersible pump to circulate water at 10 °C from the bath through the bed support. Place a 1 × 17 cm strip of Whatman No. 1 filter paper soaked with a solution of sodium hydroxide and then blotted briefly, along the cathodal side located on the left edge of the gel surface. Place a similar strip, soaked in phosphoric acid and blotted, on the anodal side, located on the right edge of the gel. Close the electrofocusing lid, taking care that the platinum wires are straight and on top of the strips of soaked papers.

Prefocus the gel at a constant voltage of 0.5 kV for 30 min. Place 0.5 × 0.5 cm squares of Whatman No. 1 filter paper on top of the gel in a straight line in the middle of the gel and about 0.5 cm apart (16 samples per plate). Apply 2.5 mL of the diluted hemolsates to the filter paper squares with a syringe (Hamilton Co., Reno, NV 89510).

Focus the samples at a constant voltage of 0.5 kV for 30 min, then increase the voltage to 1 kV for 1 h and a final voltage of 1.2 kV until the bands are clearly resolved. Transfer the focused gel to a fixing bath of trichloroacetic acid for 20 min, then rinse in a 50 mL/L glycerol solution for 1 h. Remove the filter papers and sandwich the gel between two glass plates; avoid trapping any air bubbles. Remove excess water from the gel by wiping with a disposable cloth. (Care: streaked glass will contribute to noise and erroneous baselines in the densitometry.)

Scan each sample on an Ortec 4310 densitometer at 0–3 A, using a 546.1-nm visible-bandpass filter and a slit configuration of 50 × 100 μm obtained with a 100 × 1000 μm slit mounted perpendicular to a 50 × 50 μm slit.

After scanning through each sample in three different positions, measure the peak heights (in millimeters) for the A₀ and A₁c bands and calculate the average. If present, add the peak heights of HbF, S, or C bands to the A₀ height. Calculate the percentage of HbA₁c by multiplying 100 times the height of the A₁c peak divided by the total of the peak heights (A₁c + A₀). Average the three results.

Results

Figure 1 shows a typical tracing obtained by the densitometer scanning a fixed gel after isoelectric focusing of a hemolsate from a non-diabetic adult. Resolution is near-baseline if a narrow slit is used in the photon-counting densitometer. This corresponds to a 1.5- to 2-mm separation of bands in the gel. We confirmed the position of the HbA₁c band after focusing by assaying a purified A₁c standard (7, 14). Although the HbA₂ band can sometimes be seen, it is usually too faint to show in the scan. Figure 2 shows the gradient formed by the ampholytes after isoelectric focusing. In the region of interest (pH 6–7), the gradient is 0.025 pH units per millimeter of gel surface.

We tested the linearity of the method by adding 0.22–0.83 mg of lyophilized, purified HbA₁c (14) to a hemolsate prepared from a non-diabetic adult and showed that the percentage of HbA₁c was linearly related to peak height from 5% to at least 9.3% of total hemoglobin. The range that could be tested for linearity was limited by the solubility of the HbA₁c preparation rather than the actual linear range. The within-run and between-run CV was 9.0% and 12.6% for the normal pool (mean 5.2% of total hemoglobin) and 8.4% for the above-normal pool (mean 8.5% of total hemoglobin).

We observed the migration of hemoglobin S and C in patients' samples; the resolution of the bands permits accurate quantitation of hemoglobin A₁c when the height of the S or C bands is included with A₀ in the denominator for the calculated percentage. In some samples with abnormal hemoglobins, a small peak was present just anodal to each abnormal hemoglobin band, which we believe represents the glycylated S and the glycosylated C. A hemolsate of a sample of cord blood was found to contain 66% HbF, 28% A₀, and 6% acetylated F by isoelectric focusing (Figure 1). Acetylated F migrated to the same position as HbA₁c; the HbF band was present cathodal to the A₀ band. A normal hemolsate mixed with the cord blood hemolsate is also shown in Figure 1. Interference from acetylated F is negligible in samples containing less than 20–30% HbF (as shown), because only a small fraction of the HbF is present in the acetylated form (10) that co-migrates with HbA₁c.

Figure 3 compares results for normal and diabetic hemolsates as assayed by isoelectric focusing and by cation-exchange chromatography (7). A slope of 1.03 and correlation coefficient of 0.959 indicate good agreement between the two methods. The intercept of 2.24 is consistent with the different specificities of the two tests. Two samples in this series were found to contain some hemoglobin S. Because HbS does not interfere with either method, the comparison of the results of these two samples is included in the group shown in the figure.

Results for 76 non-diabetic adults showed a slightly skewed distribution, ranging from 3.6 to 6.8% of total Hb. The observed reference interval (i.e., mean ±2 SD) was 3.7–6.1% of total
hemoglobin. The reference interval as estimated by transformation to a gaussian distribution (12) was 3.9–6.4% A1c as a percentage of total Hb. The results from 20 of these donors are shown (Figure 4) with results from adult diabetics grouped according to a physician’s rating (7, 13) of their overall control of carbohydrate metabolism (severity of disease). Several of the results from patients believed to be in “good” control overlapped the reference interval, but all of the more severely affected patients showed above-normal results for HbA1c.

The stability of HbA1c in erythrocytes was tested by comparing results obtained after storage at 30 °C for seven days with results for fresh erythrocytes from 15 non-diabetic adults. The samples were analyzed in five different runs to diminish the effect of run biases. The relationship between the two results from each sample was linear, with a slope of 0.999 and an intercept of 0.06. Results were similar for erythrocytes stored at –20 °C for seven days. Hemolysates could be stored at –70 °C for at least six months, and frozen aliquots were used as quality control pools (see above).

Discussion

Separation of HbA1c from HbA0 requires an extremely powerful technique, because they differ in only two of the more than 200 titratable groups; the difference in isoelectric point (pI) is only 0.02 pH units (15). Nevertheless, the present method is shown to be accurate, with acceptable precision.

We have incorporated the suggestion of Jeppsson et al. (16) to increase the focusing temperature from 4 °C used by some authors (17) to 10–12 °C, which improved the sharpness of the focused bands. Higher temperatures affected the gel integrity, and condensate formed above the gel on the apparatus lid. Using β-alanine as a separator to flatten the pH gradient (16) did not affect resolution in the lot of ampholyte used for Figure 1 but did improve other inferior lots. Ampholytes from Pharmacia or Bio-Rad that we tested did not provide equivalent performance. We have confirmed that good contact with the electrodes is critical (16); discontinuities appear after focusing as a wavy distortion of the bands.

Hemolysates were prepared from erythrocytes without washing because of our concern that the top layer of cells might be inadvertently removed and cause the results to be biased (18, 19). Some investigators have used a Coomassie Blue stain on the plates (6); we found that the staining contributed baseline noise in the densitometry. In our experience, the pre-poured plates supplied by LKB showed discontinuities and baseline noise. Similar problems were also found with a novel system of specially prepared cellulose acetate membranes (Jookoo Co., Ltd., Tokyo 113, Japan) soaked in ampholines as described (20).

We briefly compared gel scans obtained with the Ortec densitometer with those by a laser densitometer (Biomed Instruments, Chicago, IL 60602) and observed very low results with the laser system (16). We consider the results sufficiently precise with the photon-counting densitometer (Ortec) to obviate the need for the normalization techniques used by some authors (16). An important precaution for optimum precision in the densitometry is to be certain the A0 peak reads 70% of full scale; otherwise the A1c peak may be too small to measure reproducibly.

The high resolving power of isoelectric focusing provides certain clinical advantages over column-chromatographic and electrophoretic methods (21). Unfortunately, these other methods are not suitable for evaluation of glycoylated hemoglobin during pregnancy because hemoglobin F is included in the “glycoylated” fraction. Hemoglobin F is often present in increased concentrations in the maternal circulation, and it may appear and disappear without apparent explanation or consequence during the pregnancy (22). Therefore, a high result observed by chromatographic or electrophoretic methods for a pregnant patient may be entirely artifactual. In contrast, HbF does not interfere in the isoelectric focusing method, allowing the close monitoring of diabetic control in pregnant patients that has been often recommended. A second reflection of the increased specificity of isoelectric focusing is the observation that sample stability is less of a problem for isoelectric focusing than for other methods, because the hemoglobin degradation products that cause artifactual increases in other methods are well resolved by isoelectric focusing.

Because separate peaks can be observed on the plate, glycoylated hemoglobins S or C could be quantitatively reported from the present method. However, we believe there is insufficient clinical support for combining the analytical results into a single figure for total glycoylated hemoglobins, par-

Fig. 3. Results for 50 hemolysates as analyzed for HbA1c by isoelectric focusing and for glycohemoglobin by cation-exchange chromatography

Results are expressed as percent of total hemoglobin

Fig. 4. Percent HbA1c in hemolysates from 20 non-diabetic adults and three classes of diabetic adults grouped according to their control of carbohydrate metabolism

Horizontal lines indicate group medians. Numbers in parentheses indicate superimposed data points found in the non-diabetic group
ticularly because altered erythrocyte turnover in these hemoglobinopathies alters the clinical interpretation of glyco- 
sylated hemoglobin.

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