Hemoglobin A₁ in Hemolysates from Healthy and Insulin-Dependent
Diabetic Children, as Determined with a Temperature-Controlled Mini-
Column Assay

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We report age-specific glycohemoglobin values for non-diabetic infants, children, and young adults. These values were determined with an ion-exchange "mini-column" in a specially designed and constructed water bath that maintains column temperature at 23 ± 0.1 °C. Two in-house-prepared controls with glycohemoglobin content amounting to 6.36% and 11.87% of total hemoglobin, stored at −20 °C, were used to assess long-term analytical precision. Between-day precision (CV) was 1.4% and 1.65%, respectively. We found a significant correlation (r = 0.981, p < 0.01) between the glycohemoglobin value and the physicians' independent assessment of clinical control in 129 insulin-dependent diabetics, ages 3–23 years. There were significant differences (p < 0.05) between glycohemoglobin values between patients with well-controlled or poorly controlled diabetics and those with intermediate control. Reported studies of 20 patients over three to 12 weeks showed that changes in clinical control were paralleled by changes in glycohemoglobin values.

Additional Keyphrases: sex- and age-related effects ∙ pediatric chemistry ∙ diabetes ∙ monitoring therapy ∙ reference interval ∙ cutoff value

Within the last several years, there has been an increased acceptance of the assay of glycoylated hemoglobin in monitoring long-term blood glucose concentrations of diabetics (1–3). This acceptance has been due in part to the lack of any other reliable or convenient methods of assessing long-range blood glucose control and, therefore, of determining the relation between degree of control and the long-term sequelae associated with diabetes mellitus (4, 5). Good blood-glucose control improves the outcome of diabetic pregnancy (6, 7), but whether the long-term complications associated with diabetes are prevented by control of blood sugar has not yet been conclusively shown (8, 9). Recently, White et al. (10) reported reversal of early retinopathy in diabetics in whom blood glucose was carefully controlled, as reflected by the fact that hemoglobin A₁c was decreased to, and maintained within, the normal reference interval for one year.

In the formation of glycoylated hemoglobin, glucose is first attached as an aldimine and then undergoes an Amadori rearrangement to form a ketimine linkage (11). The former is unstable and will dissociate in media containing little or no glucose; the latter compound is stable and will not disassociate under these conditions (11). Glycoylated hemoglobin is composed of several fractions designated HbA₁ through HbA₁c. HbA₁c, HbA₁c+b+c fractions form total glycoylated hemoglobin (HbA₁c), of which HbA₁c is the major fraction, by the nonenzymatic attachment of glucose to the N-terminal valine of the beta Hb chain; HbA₁c+b+ are considered to represent artifacts (11). Many reports indicate HbA₁c is increased in diabetics but to a lesser extent than is HbA₁c. A recent study by James et al. (12) comparing HbA₁c and HbA₁c in 686 diabetic subjects revealed the two to be highly correlated (r = 0.97), with the percentage of HbA₁c = 1.18 HbA₁c + 1.67.

The major fraction of glycoylated hemoglobin (HbA₁c) was initially measured by cation chromatography, either "high-pressure" liquid chromatography (13) or by the long-column technique (14). These methods are too time-consuming, difficult, and (or) expensive for routine use in the clinical laboratory. Thus various alternative procedures have been developed for separating and measuring the total glycoylated fraction (HbA₁c): chromatographic (3, 15), electrophoretic (16, 17), and colorimetric (18, 19).

The currently most widely used of these procedures involves "mini-column" chromatography (3, 14), a procedure that has inherent problems of relatively poor precision and accuracy associated with the effect of column temperature variation (20–22). In addition, all current techniques suffer from a lack of suitable control material to assess long-term quality assurance. Temperature-correction tables (or nomograms) and commercial control specimens have not alleviated these technical difficulties.

If HbA₁c assay is to be an acceptable monitor for long-term blood glucose control, techniques used to measure it must above all demonstrate good long-term precision. This report describes a method for conveniently and accurately controlling temperature in the mini-column assay, and our development of quality control material suitable for use in routine analysis. The improved assay has been used to establish a valid reference intervals for both healthy pediatric and young-adult populations. We assessed the clinical applicability of this procedure by comparing HbA₁ values with clinical evaluation.

Materials and Methods

Hemoglobin A₁ Assay

HbA₁ was measured by using commercially available mini-columns (40 × 7 mm) of Bio-Rex 70 cation-exchanger (Isolab, Inc., Akron, OH 44321) for the chromatographic separation of hemoglobin fractions. This procedure, based on the method of Schnek and Schroeder (23), involves an erythrocyte-lysing solution (1 mL of surfactant per liter of solution), and low- and high-concentration phosphate–cyanide buffers for eluting the hemoglobin fractions separately. Fifty microliters of whole blood is lysed with 200 μL of the surfactant solution. Fifty microliters of this hemolysate is applied to the mini-columns (prepared according to the manufacturer’s instructions) and allowed to drain into the columns. Following a 0.2-mL wash of the column with a low-phosphate buffer, 4 mL of the buffer is used to elute the glycoylated ("fast") hemoglobin fraction from the column. Next, 4 mL of the high-concentration phosphate–cyanide buffer is

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applied to the column, to elute the remaining hemoglobin fraction. This fraction ("slow") is diluted with 16 mL of distilled water. The absorbance at 415 nm (A415) is measured for both fractions, and the percentage glycated hemoglobin (% HbA1) is calculated as follows:

$$\text{% HbA1} = \frac{A_{415}^{\text{fast}} + (5 \times A_{415}^{\text{slow}})}{1} \times 100$$

We modified this procedure by assaying with temperature strictly controlled by use of a Plexiglas water-bath unit, constructed to hold as many as 20 mini-columns during the chromatographic development (Figure 1). The design of this water bath allows convenient access to both ends of the column during the chromatographic assay while water is circulated by a constant temperature bath (Haake, Saddlebrook, NJ 07662). The range for column temperature was 22.9 to 23.1 °C.

**Analysis at Ambient vs Stringently Controlled Temperature**

Venous blood samples from non-fasting subjects were collected in 2-mL EDTA-containing Vacutainer Tubes (Becton Dickinson Co., Rutherford, NJ 07070) and refrigerated for 2 to 3 h before analysis. The specimens were analyzed for HbA1 at the four different ambient temperatures in different locations in the laboratory. Results obtained under these conditions were corrected to results at 23 °C by use of a temperature correction table supplied by the column manufacturer (Isolab, Inc.). At the same time each specimen was analyzed in quadruplicate at 23 ±0.1 °C in the temperature-controlled water bath.

**Quality-Control Material**

EDTA-treated plasma specimens were collected from a non-diabetic laboratory volunteer with a normal proportion of HbA1 and from a diabetic subject known to have an above-normal value for HbA1. The erythrocytes were washed twice with 9.0 g/L NaCl solution, the volume was reconstituted in saline solution to what it was in the original blood sample, and a hemolysate was prepared in the same proportions as for the column analysis (i.e., 50 parts of mixed erythrocytes to 200 parts of lysing solution). The lysate was extracted twice with chloroform to remove membranous material. The extracted hemolysate was placed in washed dialysis tubing (Spectrapor; Spectrum Medical Industries, Inc., Los Angeles, CA 90064) and dialyzed at 4 °C vs a phosphate-cyanide buffer (4.59 g of monosodium phosphate monohydrate, 1.18 g of disodium phosphate, and 0.65 g of potassium cyanide per liter of water, adjusted to pH 6.70 ± 0.12) containing 1 mg of chloramphenicol per liter and equilibrated with carbon monoxide gas. The dialysis was continued for five days, with a change of dialyzing fluid each day. After dialysis, the lysate was removed and centrifuged at 1300 rpm (600 × g) at room temperature for 15 min to remove any precipitated protein. The clear hemoglobin solution was then frozen in small aliquots at −20 °C, forming a normal and an abnormal pool ("IFC 3" and "IFC 4," respectively).

Pre-assayed lyophilized hemoglobin control material was obtained from Isolab, Inc. The lyophilized hemoglobin was reconstituted according to the manufacturer's instructions and refrigerated between use. Individual vials of each lot were analyzed for up to five days after reconstitution.

**Patient Analysis**

Two separate populations were studied with respect to their glycohemoglobin concentrations: (a) 167 non-diabetic infants, children, and young adults, ages one week to 30 years; (b) 125 insulin-dependent diabetics, ages 3–23 years. For 20 of the latter group, determinations of HbA1 were repeated during three to 12 weeks. In all cases, the specimens were collected in tubes containing potassium EDTA and stored at 4–5 °C for not longer than 24 h before analysis.

At the time HbA1 was measured, all patients in the diabetic group were also assessed with respect to their degree of clinical control. This assessment, performed by the clinic physician in consultation with other members of the diabetic-management team, included several factors: urine glucose pattern, hypoglycemic episodes, diabetic ketoacidosis, ketonuria, nocturia, polyuria, weight loss, growth on percentile charts, and therapeutic compliance. For each factor either no points or one point was assigned, except for glycosuria, where two points were given for persistent values of 3% or greater, one point for 1–2%, and 0 points for persistently negative urines. In this manner, a final score ranging from 1 to 10 points was assigned. Thus, a patient judge to be in excellent control since the previous clinic visit would receive a rating of 1 to 2, while one assessed as being in very poor control would be assigned a rating of 9 to 10. This evaluation process was repeated at all subsequent clinical visits at which blood was taken for HbA1 estimation. At no time before the HbA1 assay was completed for a particular patient was the laboratory aware of the clinical rating; in turn, the rating was always completed before the HbA1 result was seen by the clinic physicians.

**Statistical Analysis**

An analyses were performed with an Amdahl 470 V/6 computer, using an established statistical software package (Statistical Analytical Systems Institute, Raleigh, NC). Differences between variously rated clinical control groups were tested for statistical significance by analysis of variance.

**Results**

**Method Assessment**

To assess the effect of temperature on the HbA1 procedure, we assayed six patients' samples at various ambient temperatures (20–31 °C) and at the controlled 23 ± 0.1 °C. Results for HbA1 obtained at ambient temperatures were converted back to 23 °C by using a chart supplied by Isolab, Inc. (Table 1).

The CV for quadruple analysis of each sample at 23 ± 0.1 °C in the water bath ranged from 1.8 to 4.5%. The CV for the ambient-run corrected values ranged from 9.5 to 18%. The mean ambient-run corrected values for the HbA1 were consistently lower than those for samples in the water bath at 23 ± 0.1 °C (Table 1). A further 35 patients' samples were analyzed during 10 days, once at 23 ± 0.1 °C in the water-bath

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Fig. 1. Temperature-controlled water bath for HbA1 chromatography
system, and once at the prevailing ambient temperature in the laboratory. To determine the relation between column temperature and the change in glycohemoglobin, we divided the values obtained under water-bath controlled and ambient-temperature conditions by the difference in temperature between the two columns for each patient's sample, i.e., Δ HbA1C + Δ temp. to obtain a factor expressed as %HbA1C. For the 35 patients, this factor, which had a mean and standard deviation of 0.344 ± 0.19, was applied to the uncorrected values obtained for the six samples in Table 1 and a second set of HbA1C values, corrected to 23 °C, was derived (Table 1).

Two different lots of commercial lyophilized controls for HbA1C were reconstituted daily and analyzed at 23 °C for up to five days after reconstitution. The mean HbA1C value for each lot was greater by the second (lot no. 4281) or third (lot no. 6230) day after reconstitution (Table 2). The CV for each day of analysis ranged from 1.4 to 5.7%. An aliquot of the normal “in-house” frozen control (IFC 3), thawed and assayed on each day, showed excellent stability (Table 2). The CV for IFC 3, analyzed on each of 15 working days, was 1.4%. The increased “in-house” HbA1C pool (IFC 4), similarly examined, showed a between-day CV of 1.65% (n = 20).

These two pools have been in routine use for six months with maintenance of CVs of <2% for both pools.

### Clinical Assessment

We used the temperature-controlled procedure and the “in-house” frozen hemolysates (IFC 3 and 4) as a monitor of quality assurance throughout the clinical-assessment studies.

HbA1C reference intervals were established for a population of non-diabetic children and young adults. These values (Table 3) show markedly higher values for children below the age of six months, with a subsequent, gradual reduction in HbA1C values until the age of four years. Several high-value samples were analyzed by electrophoresis, which confirmed the presence of significant amounts of HbF. From five to 30 years, the mean HbA1C values remained stable at about 7% of total Hb (Table 3). No statistically significant sex- or race-related differences were found at any age.

#### Table 1. HbA1C (% of Total Hb) In Six Patients' Samples Run at 23 ± 0.1 °C and at Various Ambient Room Temperatures and Corrected to 23 °C

<table>
<thead>
<tr>
<th>Water bath °C</th>
<th>Range</th>
<th>Ambient °C: HbA1C corrected to 23 °C; run at</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>(5.86-6.15)</td>
<td>7.0</td>
</tr>
<tr>
<td>6.0</td>
<td>(5.72-6.35)</td>
<td>5.7</td>
</tr>
<tr>
<td>3.8</td>
<td>(3.65-3.93)</td>
<td>4.8</td>
</tr>
<tr>
<td>3.8</td>
<td>(6.3-6.6)</td>
<td>6.2</td>
</tr>
<tr>
<td>5.9</td>
<td>(5.78-6.21)</td>
<td>5.6</td>
</tr>
<tr>
<td>6.4</td>
<td>(7.8-8.14)</td>
<td>6.1</td>
</tr>
<tr>
<td>7.9</td>
<td>(7.3-7.9)</td>
<td>5.5</td>
</tr>
</tbody>
</table>

*a* The top value in each pair is the value obtained after correction according to the supplier's (Isolab) nomogram. The bottom value is the value obtained after use of our derived correction factor (0.344 % HbA1C/°C). *b* Based on four assays.

#### Table 2. Between-Day Precision (Mean, and Range of HbA1C) as % of Total) for Commercial Lyophilized Controls (CLC) and “In-House” Frozen Controls (IFC)

<table>
<thead>
<tr>
<th>Day of reconstitution/ thawing</th>
<th>n</th>
<th>Second day post-reconstitution</th>
<th>n</th>
<th>Third day post-reconstitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLC 4281</td>
<td>10</td>
<td>7.86 (7.5-8.4)</td>
<td>8</td>
<td>8.36 (7.6-9.0)</td>
</tr>
<tr>
<td>CLC 6230</td>
<td>9</td>
<td>7.11 (6.7-7.4)</td>
<td>9</td>
<td>7.14 (7.0-7.3)</td>
</tr>
<tr>
<td>IFC 3</td>
<td>15</td>
<td>6.36 (6.1-6.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFC 4</td>
<td>20</td>
<td>11.87 (11.48-12.21)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n, no. analyses (on n different days).

#### Table 3. HbA1C Values (% of Total Hb) in 179 Non-Diabetic “Reference” Subjects

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>&lt;3/12</th>
<th>&lt;6/12</th>
<th>&lt;1</th>
<th>&lt;2</th>
<th>&lt;4</th>
<th>5-12</th>
<th>13-20</th>
<th>21-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>8</td>
<td>5</td>
<td>13</td>
<td>11</td>
<td>20</td>
<td>45</td>
<td>58</td>
<td>19</td>
</tr>
<tr>
<td>Mean, %</td>
<td>64.1*</td>
<td>14.4</td>
<td>10.0</td>
<td>9.2</td>
<td>7.7</td>
<td>7.1</td>
<td>7.1</td>
<td>6.5</td>
</tr>
<tr>
<td>SD, %</td>
<td>13.9</td>
<td>4.6</td>
<td>2.3</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*a* These high values are the result of interference by HbF, and therefore unreliable (see text).
HbA₁ was then measured in 129 diabetics ranging in age from three to 23 years, and correlated with the degree of clinical control assessed at that clinic visit as previously described (Figure 2).

Differences in mean HbA₁ between clinical groups were assessed by one-way analysis of variance (Table 4). The normal population and the diabetic group differed significantly (p < 0.001). The normal group and the best-controlled diabetic group (groups 1–2) differed less, but still significantly (p < 0.05). Significant differences (p < 0.001) were found between group 1–2 and all other clinical groups (Table 4) except group 3–4. Similarly, mean HbA₁ values in group 3–4 differ significantly (p < 0.001) from groups 5–10. However, no or little significant differences in HbA₁ values were found between groups 5–8. Clinical group 9–10 had HbA₁ values that differed significantly (p < 0.001) from those for all other clinical groups (Table 4).

Twenty cases were followed for three to 12 weeks before a repeat clinical assessment and HbA₁ measurement (Figure 3). In 15 of these patients, the change in clinical control was reflected by a similar change in HbA₁ values, that is, improved clinical control with lower HbA₁ or deteriorating control with higher HbA₁ values. In four patients, maintenance of clinical control between visits was associated with virtually no change in HbA₁ values (Figure 3). Only in one patient was a large increase in HbA₁ values (10.6 to 15.3%) associated with no change in assessment of clinical control grouping.

**Discussion**

When a method that will be used for monitoring changes or therapy in any disease state is being established, a high degree of analytical precision is necessary in addition to reasonable accuracy. This is especially pertinent in chronic disorders where therapeutic and clinical complications are to be assessed over many years, as in the case of diabetes. Furthermore, with the recent report by White et al. (10) that tight control of diabetes as judged by HbA₁ values below 8% will cause reversal of early ocular abnormalities, long-term precision in HbA₁ measurements appears even more important.

The accuracy of the “mini-column” procedure, as compared to the original “long-column” procedure of Trivelli et al. (14), has been previously documented when the assays are operated under similar conditions (3, 13). However, the precision of the mini-column method has recently been called into question, especially as less temperature-dependent methods have been developed (21, 22). As demonstrated by us and others (20–24), lack of column temperature control during analysis reduces precision.

If the temperature in the mini-column procedure can be

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**Table 4. One-Way Analysis of Variance (F Values) for HbA₁ between Groups of Diabetic Children under Various Degrees of Clinical Control**

<table>
<thead>
<tr>
<th>Clinical control groups</th>
<th>Normal</th>
<th>1–2</th>
<th>3–4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9–10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>—</td>
<td>4.24*</td>
<td>13.3†</td>
<td>118.3†</td>
<td>111.5†</td>
<td>242.2†</td>
<td>190†</td>
<td>403.1†</td>
</tr>
<tr>
<td>1–2</td>
<td>—</td>
<td>2.3</td>
<td>31.1†</td>
<td>37.4†</td>
<td>65.5†</td>
<td>64.3†</td>
<td>154.9†</td>
<td></td>
</tr>
<tr>
<td>3–4</td>
<td>—</td>
<td>10.6†</td>
<td>15.4†</td>
<td>28.8†</td>
<td>30.6†</td>
<td>89.8†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>1.2</td>
<td>7.2†</td>
<td>9.27†</td>
<td>65.8†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>1.6</td>
<td>3.0</td>
<td>40.1†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>0.5</td>
<td>36.0†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>23.3†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9–10</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05, † p < 0.01, ‡ p < 0.001
Table 5. Relation between Degree of Clinical Control and Suggested HbA1c "Cutoff" Values

<table>
<thead>
<tr>
<th>Clinical control groups</th>
<th>HbA1c, %</th>
<th>1-2</th>
<th>3-4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9-10</th>
<th>% of total group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent control</td>
<td>&lt;9</td>
<td>82</td>
<td>12</td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>—</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>Good control</td>
<td>&gt;9 &lt;11</td>
<td>6</td>
<td>58</td>
<td>40</td>
<td>46</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Fair control</td>
<td>&gt;11 &lt;13</td>
<td>—</td>
<td>18</td>
<td>28</td>
<td>18</td>
<td>44</td>
<td>55</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>Poor control</td>
<td>&gt;13 &lt;15</td>
<td>6</td>
<td>6</td>
<td>16</td>
<td>18</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Very poor control</td>
<td>&gt;15</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>12</td>
<td>3</td>
<td>15</td>
<td>50</td>
<td>14</td>
</tr>
</tbody>
</table>

No. in group

16  15  25  15  31  21  20  143

be a significant clinical problem in assessing the level of control in patients who are not either extremely well or extremely poorly controlled (groups 1–2 or 9–10, respectively). Table 5 shows the clinical population distributed according to their HbA1c values. Even in groups 1–2 and 9–10, nearly 20% of each group would have been placed in either a worse or better control group, respectively, if HbA1c values had been the determinant. In addition, less than 30% of the patients fell into these two somewhat clearly defined clinical groupings; most were in control groups 3–8 and had HbA1c values ranging from <9% to >15%. Evidently, precise HbA1c analysis, with use of stable control material upon which to assess long-term precision and an appropriate set of reference intervals, can provide the diabetologist, and in turn the diabetic, with a useful and reliable index upon which to set therapeutic goals and monitor progress.

References

15. Welch, S. G., and Boucher, B. J., A rapid micro-scale method for


