Agar Gel Electrophoretic Determination of Glycosylated Hemoglobin: Effect of Variant Hemoglobins, Hyperlipidemia, and Temperature

H. Aleyassine, R. J. Gardiner, L. A. Blankstein, and M. E. Dempsey

We investigated the effect of temperature, variant hemoglobins, and hyperlipidemia on determination of glycosylated hemoglobin by an electrophoretic method (Clin. Chem. 26: 1598–1602, 1980). We found that: (a) temperature variations ranging from 4 to 30 °C were without effect on results obtained by electrophoresis; (b) concurrent determination of glycosylated hemoglobin by electrophoresis and column-chromatography in blood specimens from 150 diabetic patients yielded almost identical mean values for both procedures when procedures were carried out at 22 °C; (c) electrophoretic determination of glycosylated hemoglobin in whole-blood hemolysate was not affected by concentration of triglycerides; and (d) unlike column-chromatographic procedures, which underestimate the percentage of glycosylated hemoglobin in patients with hemoglobin S and C, the electrophoretic method accurately determined the proportion of glycosylated hemoglobin in these hemoglobinopathies. Evidently, electrophoresis on agar gel is an excellent alternative to cation-exchange column-chromatographic methods for glycosylated hemoglobin.

Additional Keyphrases: hemoglobinopathy • HbS • HbC • screening • monitoring diabetes

Cation-exchange column-chromatographic methods are being used increasingly for quantitative determination of glycosylated hemoglobin (GHB). Although, in general, these procedures appear to be suitable for routine clinical use, they consistently underestimate the percentage of GHB in the two most common hemoglobinopathies, HbS and HbC (1). In addition, several other factors such as hyperlipidemia (2), fluctuations in ambient temperature (3), and minor variations in pH or ionic strength of the buffer solutions (4, 5) also interfere with accurate determination of GHB by column chromatography. An alternative method is evidently needed.

We recently described (6) an agar-gel electrophoretic method for determination of GHB, involving a system derived from the initial work of Allen et al. (7). The method proved to be highly reproducible and results correlated well with those by liquid chromatography (6).

The present study is an extension of our previous work. We have further investigated the effect of HbS and HbC, hyperlipidemia, and a wide range of temperature on the electrophoretic determination of GHB. We have also correlated electrophoretic results for a large number of diabetic patients with those obtained by use of a commercially available microcolumn-chromatographic kit.

Materials and Methods

All patients were seen at the Diabetic Center of the Montreal General Hospital. For GHB determinations, blood was collected in a tube with EDTA anticoagulant and tested the same day. We used two basically different systems—electrophoresis and cation-exchange column-chromatography—to quantify GHB. The column-chromatographic method, that of Isolab, Akron, OH 44321, was selected because of our extensive experience with this system (1, 8, 9). The electrophoretic method was as recently described (6), except that electrophoresis time was prolonged from 40 min to 50 min, a change essential for clear separation of glycosylated HbC from non-glycosylated HbC. All necessary materials and equipment for electrophoresis were provided by Corning Medical, Medfield, MA 02052. Serum triglycerides were measured enzymatically with an Abbott Bichromatic Analyzer-100, with use of the Abbott A-Gent Triglycerides reagent system.

Results

Under conditions of the present studies, when hemolysates from patients with normal hemoglobin (HbA) are subjected to electrophoresis, two distinct hemoglobin components can be identified: a fast minor fraction (HbA1) and a slow major fraction (HbA0), both migrating cathodic to the point of application. Identification of the fast fraction as HbA1 was based on the following criteria: (a) the incubation of erythrocytes or hemolysates in the presence of high concentrations of dextrose (25–50 mmol/L) for 4–8 h at 37 °C resulted in a significant increase in the percentage of HbA1c (fast hemoglobin) and a corresponding decrease in the percentage of HbA0c (slow hemoglobin); (b) concurrent determination at 22 °C of HbA1c in blood specimens from 150 ambulatory diabetic patients by a microcolumn chromatographic method (Isolab) and by electrophoresis yielded almost identical mean (and SD) values

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2 Corning Medical, Medfield, MA 02052.

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for both procedures (column chromatography, 12.47 ± 3.05%; electrophoresis, 12.43 ± 3.17%); (c) HbA, values obtained by electrophoresis and by column chromatography were strongly correlated (r = 0.93) (Figure 1).

Effect of temperature on GHB determination by column-chromatography and electrophoresis. In a comparative study we measured the percentage of GHB in eight different blood samples by the two methods, at various ambient temperatures. At each designated temperature, buffer solutions and columns were equilibrated before operations. The results show that temperature variations ranging from 4 to 30 °C are without effect on GHB determinations by electrophoresis, but the same temperature variations are accompanied by considerable changes in GHB values obtained by chromatography (Table 1). Note that at 22 °C the two techniques yielded similar results.

Electrophoretic determination of glycoylated hemoglobin S (GHSB) and glycoylated hemoglobin C (GHBC). Figure 2 shows the hemoglobin electrophoretic pattern of blood specimens from patients with HbA, HbAS, and HbAC. As can be seen, unlike normal hemoglobin (HbA), hemolysates of patients heterozygous for HbS and HbC separate into four distinct fractions. These fractions are identified as A1, S1, A0, and S0, for blood samples containing HbAS; and A1, A0, C1, and C0, for blood specimens with HbAC. Figures 3 and 4 show the electrophoretic distribution of HbAS and HbAC components as recorded by scanning densitometry at 420 nm.

In a preceding paper (6) we tentatively designated S1 and C1 as GHBS and GHBC, respectively. To confirm that S1 and C1 are indeed the glycoylated forms of HbS and HbC, in a follow-up study we monitored the percentage of various hemoglobin components in AS and AC patients subjected to three different modes of therapy for diabetes (insulin, oral hypoglycemic agents, and diet alone). As a result of intensive therapy with insulin and oral hypoglycemic agents, a significant decrease in S1 and C1 was noted after three months of treatment (Tables 2 and 3). A decline proportional to the decrease in the percentage of HbA1 in each patient. Finally, in all cases the ratio of A1/(A0 + A1) was similar to that of S1/(S0 + S1) or C1/(C0 + C1), both at the beginning and at the end of the study (Tables 2 and 3).

Figure 3. Electrophoretic distribution of hemoglobin components in a blood sample containing HbAS, as recorded by scanning densitometry at 420 nm.

Table 1. Effect of Temperature on Glycoylated Hemoglobin Determined by Two Techniques

<table>
<thead>
<tr>
<th>Temp, °C</th>
<th>Column chromatography</th>
<th>Electrolysis</th>
<th>GHB, as percent of total Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16</td>
<td>22</td>
<td>30</td>
</tr>
<tr>
<td>4.6</td>
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<td>9.7</td>
<td>16.8</td>
<td>28.5</td>
<td>16.6</td>
</tr>
<tr>
<td>9.0</td>
<td>17.3</td>
<td>27.6</td>
<td>18.3</td>
</tr>
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<td>9.6</td>
<td>16.5</td>
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<td>7.6</td>
<td>12.8</td>
<td>22.9</td>
<td>12.7</td>
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<td>5.3</td>
<td>8.3</td>
<td>16.4</td>
<td>8.4</td>
</tr>
<tr>
<td>7.5</td>
<td>11.6</td>
<td>21.2</td>
<td>11.2</td>
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<tr>
<td>9.2</td>
<td>16.2</td>
<td>28.4</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Table 2 shows the hemoglobin electrophoretic pattern of blood specimens from patients with HbA, HbAS, and HbAC. As can be seen, unlike normal hemoglobin (HbA), hemolysates of patients heterozygous for HbS and HbC separate into four distinct fractions. These fractions are identified as A1, S1, A0, and S0, for blood samples containing HbAS; and A1, A0, C1, and C0, for blood specimens with HbAC. Figures 3 and 4 show the electrophoretic distribution of HbAS and HbAC components as recorded by scanning densitometry at 420 nm.

In a preceding paper (6) we tentatively designated S1 and C1 as GHBS and GHBC, respectively. To confirm that S1 and C1 are indeed the glycoylated forms of HbS and HbC, in a follow-up study we monitored the percentage of various hemoglobin components in AS and AC patients subjected to three different modes of therapy for diabetes (insulin, oral hypoglycemic agents, and diet alone). As a result of intensive therapy with insulin and oral hypoglycemic agents, a significant decrease in S1 and C1 was noted after three months of treatment (Tables 2 and 3). A decline proportional to the decrease in the percentage of HbA1 in each patient. Finally, in all cases the ratio of A1/(A0 + A1) was similar to that of S1/(S0 + S1) or C1/(C0 + C1), both at the beginning and at the end of the study (Tables 2 and 3).

Effect of hyperlipidemia on GHB determination by column-chromatography and electrophoresis. Blood specimens
containing various concentrations of triglycerides were obtained from diabetic patients and used for the measurement of GHb by microcolumn-chromatography and electrophoresis. To evaluate the effect of hyperlipidemia on these determinations, we used saline-washed erythrocytes from the same specimens as the control. As shown in Table 4, the column-chromatographic method overestimated the percentage of GHb in whole-blood hemolysate when serum triglyceride concentrations exceeded 8 g/L. This spurious increase appeared to be proportional to the serum concentrations of triglycerides. However, GHb values obtained by electrophoresis remained unaffected throughout the range of triglyceride concentrations tested.

**Discussion**

Earlier (6) we showed an excellent correlation \( r = 0.98 \) between electrophoretic values for GHb and those obtained by liquid chromatography. In the present study we similarly have compared electrophoresis and the Isolab microcolumn-chromatographic method and found a strong correlation \( r = 0.93 \) between the two. Furthermore, the mean GHb values were almost identical for electrophoresis and column chromatography.

A major difficulty in measurement of GHb by cation-exchange column-chromatography is the extreme sensitivity of the resin to variations in ambient temperature. As a result, manufacturers of microcolumn kits recommend that GHb be determined at about 22 °C, because the values obtained at this temperature best reflect the actual percentage of GHb in the blood specimens. For many laboratories, however, it is impractical to maintain a constant ambient temperature throughout the year for just one test. To remedy this situation, some commercial laboratories now provide a temperature-correction chart with their kits.

To evaluate the effect of temperature on GHb determinations by electrophoresis and column chromatography, we monitored the apparent percentage of GHb at various ambient temperatures and found that at 22 ± 0.5 °C similar results are indeed obtained by both procedures. Our results also show that unlike column-chromatographic values, which are considerably affected by temperature variations, the values obtained by electrophoresis are insensitive to these changes.

**Table 2. A Three-Month Follow-up Study of GHb Percentages in Six Patients with Hemoglobin AS**

<table>
<thead>
<tr>
<th>Therapy</th>
<th>( A_1 )</th>
<th>( S_1 )</th>
<th>( A_0 )</th>
<th>( S_0 )</th>
<th>( \frac{A_1}{(A_0 + A_1)} )</th>
<th>( S_1/(S_0 + S_1) )</th>
<th>( A_1 )</th>
<th>( S_1 )</th>
<th>( A_0 )</th>
<th>( S_0 )</th>
<th>( \frac{A_1}{(A_0 + A_1)} )</th>
<th>( S_1/(S_0 + S_1) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>9.0</td>
<td>6.3</td>
<td>49.9</td>
<td>34.8</td>
<td>15.2</td>
<td>15.3</td>
<td>6.5</td>
<td>4.2</td>
<td>52.8</td>
<td>36.4</td>
<td>10.9</td>
<td>10.3</td>
</tr>
<tr>
<td>Insulin</td>
<td>12.3</td>
<td>7.7</td>
<td>48.3</td>
<td>31.7</td>
<td>20.2</td>
<td>19.5</td>
<td>8.6</td>
<td>5.6</td>
<td>51.9</td>
<td>33.9</td>
<td>14.2</td>
<td>14.1</td>
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<tr>
<td>OHA</td>
<td>9.5</td>
<td>6.4</td>
<td>51.1</td>
<td>33.0</td>
<td>15.6</td>
<td>16.2</td>
<td>7.2</td>
<td>4.7</td>
<td>54.6</td>
<td>33.5</td>
<td>11.6</td>
<td>12.3</td>
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<tr>
<td>OHA</td>
<td>8.2</td>
<td>5.8</td>
<td>50.8</td>
<td>35.2</td>
<td>13.8</td>
<td>14.1</td>
<td>6.3</td>
<td>4.8</td>
<td>50.4</td>
<td>38.7</td>
<td>11.1</td>
<td>10.6</td>
</tr>
<tr>
<td>Diet</td>
<td>6.1</td>
<td>3.8</td>
<td>56.7</td>
<td>33.4</td>
<td>9.7</td>
<td>10.2</td>
<td>6.4</td>
<td>4.2</td>
<td>53.2</td>
<td>36.2</td>
<td>10.7</td>
<td>10.3</td>
</tr>
<tr>
<td>Diet</td>
<td>6.4</td>
<td>3.6</td>
<td>58.3</td>
<td>31.7</td>
<td>9.8</td>
<td>10.1</td>
<td>6.7</td>
<td>3.8</td>
<td>56.0</td>
<td>33.5</td>
<td>10.6</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Values for \( A_1, S_1, A_0, \) and \( S_0 \) are expressed as percent of total Hb in the hemolysate. OHA refers to oral hypoglycemic agent.

**Table 3. A Three-Month Follow-up Study of GHb Percentages in Four Patients with Hemoglobin AC**

<table>
<thead>
<tr>
<th>Therapy</th>
<th>( A_1 )</th>
<th>( A_0 )</th>
<th>( C_1 )</th>
<th>( C_0 )</th>
<th>( \frac{A_1}{(A_0 + A_1)} )</th>
<th>( C_1/(C_0 + C_1) )</th>
<th>( A_1 )</th>
<th>( A_0 )</th>
<th>( C_1 )</th>
<th>( C_0 )</th>
<th>( \frac{A_1}{(A_0 + A_1)} )</th>
<th>( C_1/(C_0 + C_1) )</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>12.2</td>
<td>43.8</td>
<td>9.4</td>
<td>34.6</td>
<td>21.7</td>
<td>21.3</td>
<td>8.5</td>
<td>49.6</td>
<td>6.3</td>
<td>35.6</td>
<td>14.6</td>
<td>15.0</td>
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<tr>
<td>Insulin</td>
<td>9.7</td>
<td>47.9</td>
<td>7.4</td>
<td>35.0</td>
<td>16.8</td>
<td>17.4</td>
<td>6.5</td>
<td>52.4</td>
<td>4.7</td>
<td>38.4</td>
<td>11.0</td>
<td>11.4</td>
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<tr>
<td>Diet</td>
<td>6.8</td>
<td>58.3</td>
<td>3.9</td>
<td>31.1</td>
<td>10.4</td>
<td>11.1</td>
<td>6.8</td>
<td>57.0</td>
<td>4.1</td>
<td>32.1</td>
<td>10.6</td>
<td>11.3</td>
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<tr>
<td>Diet</td>
<td>5.8</td>
<td>53.8</td>
<td>3.6</td>
<td>37.0</td>
<td>9.4</td>
<td>8.8</td>
<td>5.7</td>
<td>54.2</td>
<td>3.9</td>
<td>36.2</td>
<td>9.5</td>
<td>9.7</td>
</tr>
</tbody>
</table>

Values for \( A_1, A_0, C_1, \) and \( C_0 \) are expressed as percent of total Hb in the hemolysate.

**Table 4. Effect of Hyperlipidemia on Glycosylated Hemoglobin Determination by Two Techniques**

<table>
<thead>
<tr>
<th>Serum triglycerides, g/L</th>
<th>Column-chromatography</th>
<th>Electrophoresis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Whole-blood erythrocytes</td>
<td>Washed erythrocytes</td>
</tr>
<tr>
<td>GHb (% of total Hb)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75.00</td>
<td>19.8</td>
<td>13.6</td>
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<tr>
<td>64.00</td>
<td>19.2</td>
<td>13.7</td>
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<td>50.00</td>
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<td>38.00</td>
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<td>24.00</td>
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<td>11.00</td>
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<td>14.4</td>
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<tr>
<td>8.00</td>
<td>16.3</td>
<td>16.1</td>
</tr>
</tbody>
</table>

Fig. 4. Electrophoretic distribution of hemoglobin components in a blood sample containing HbAC, as recorded by scanning densitometry at 420 nm

\( A_1 = 6.2\% ; A_0 = 51.4\% ; C_1 = 5.4\% ; C_0 = 35.0\% \)

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Previous studies have shown that cation-exchange column-chromatographic procedures underestimate the percentage of GHb in patients heterozygous for HbS or HbC (1, 8), and that the correction of these spuriously low values necessitates prior determination of the total (glycosylated and non-glycosylated) abnormal hemoglobins by an alternative method. In contrast to column-chromatographic systems, the electrophoretic method described here is a one-step procedure in which GHbS and GHbC are determined directly. An additional advantage of the method is its ability to reveal and measure HbS and HbC in patients not suspected of having a hemoglobinopathy, while determining the percentage of GHb. Obviously, this latter feature is of considerable clinical significance.

The results of our clinical follow-up studies also revealed that in each patient the variations in the percentage of GHbS or GHbC paralleled those of HbA1. These observations confirm the results of the previous studies (1, 8, 10) indicating that HbS and HbC are indeed subjected to the glycosylation process, and that the rate of glycosylation of these variant hemoglobins is similar to that of normal hemoglobin.

Hyperlipidemia is a relatively common feature of diabetes mellitus. When column-chromatographic methods are used for the determination of GHb in the whole-blood hemolysate, the lipid components elute with the HbA1 fraction and produce a turbid solution. This turbidity interferes with the absorbance readings of GHb, causing falsely high values (2). In the present study we examined the effect of hyperlipidemia on the electrophoretic and column-chromatographic determinations of GHb. The results have shown that only chromatographic values were affected by high concentrations of triglycerides in the blood.

We conclude that electrophoresis on agar gel offers an excellent alternative to cation-exchange column chromatography for determination of GHb.

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References