New Spectrophotometric Method for the Determination of Hemoglobin A1c Compared with a Microcolumn Technique

Olov Wälinder, Gunnar Ronquist, and Pehr-Johan Fager

We compared a spectrophotometric kit method (Glycospec) for determination of glycosylated hemoglobin (HbA1c) with a microcolumn kit method (Bio-Rad). The Glycospec method is based on the change in absorbance when phytic acid binds to hemoglobin A. With glycosylated hemoglobin there is no such change because the binding is blocked by the sugar moiety. Inter-assay CVs were 2–6% for both methods. In healthy subjects the mean (±SD) value for HbA1c was about 1% higher with the spectrophotometric than the microcolumn method. For samples from 122 diabetics the correlation between values for HbA1c obtained with the two methods was acceptable (r = 0.89), although the spectrophotometric technique yielded 2–4% higher values, a difference at least partly due to the absence of 2,3-diphosphoglycerate from the spectrophotometric standards. Adding 1.8 mmol of it per liter to these standards caused displacement of the standard curve; HbA1c values then agreed well with those of the microcolumn method. The spectrophotometric procedure is easily automated, and therefore is well suited for large-scale analyses if problems with standards and calibration can be solved.

Additional Keyphrases: monitoring diabetes • chromatography, liquid • "kit" methods

Glycosylated hemoglobin (hemoglobin A1) normally comprises about 6% of the total hemoglobin of the erythrocyte. The predominant subfraction of hemoglobin A1 (HbA1) is HbA1c, formed by a slow non-enzymic glycosylation reaction during the life-time of the erythrocyte (1). Thus the percentage of HbA1c reflects the average blood glucose concentration during the preceding four or five weeks (2, 3). Both HbA1c and the total HbA1 fraction are increased in diabetes mellitus, and determination of HbA1c has become an important index of long-term diabetic control (4–6). The method most widely used for determining HbA1c is based on chromatography through microcolumns (7, 8)—a method not ideally suited for routine clinical use because it is difficult to automate and is highly sensitive to minor changes in experimental conditions (9). Recently, a spectrophotometric assay based on a new principle was reported (10). This paper presents a comparison of the two methods with regard to reliability of results for normal subjects and diabetic patients. Various factors influencing the HbA1c values were also studied.

Materials and Methods

The control group consisted of 18 ostensibly healthy blood donors, most of them men, with no clinical signs of diabetes mellitus. The 122 diabetic patients were regularly attending our outpatient service for diabetic care. They were being treated with insulin, sulfonylurea drugs, or diet alone. Blood samples for HbA1c determinations, obtained after an overnight fast, were drawn in EDTA-containing tubes, unless otherwise stated. They were analyzed concomitantly by the microchromatographic and the spectrophotometric methods.

HbA1c analysis by the use of microcolumns was performed with a commercial kit (Bio-Rad Laboratories, Richmond, CA 94804). Blood was drawn into EDTA-containing tubes, if not otherwise stated, and was mixed with a hemolyzing reagent. An aliquot of each hemolysate was applied to the microcolumn. Adding the elution buffer rapidly eluted HbA1c from the column, while the main part of the hemoglobin was retarded. The percentage of HbA1c in the blood samples was determined by measuring the absorbance at 415 nm of the eluted HbA1c fraction and the diluted hemolysate. The eluant buffer was kept at 22 °C, the temperature being continuously monitored with a microelectrode inserted into the resin. Values deviating from 22 °C were corrected according to a nomogram provided by the manufacturer.

The spectrophotometric assay is based on the fact that organophosphorus compounds such as 2,3-diphosphoglycerate (2,3-DPG) and inositol hexaphosphate (phytic acid) bind to the N-terminal amino acid of the two beta chains of hemoglobin A (10). This causes a conformational change of the hemoglobin molecule, a process that can be monitored spectrophotometrically. The prerequisite for such a change is available N-terminal residues. Hence, the spectrum of glycosylated hemoglobin is not changed by the organophosphorus compounds because of the blocking effect of the glucose moiety. The change in absorbance induced by phytic acid is thus inversely proportional to the percentage of glycosylated hemoglobin.

The spectrophotometric assay was done in accordance with the manufacturer's instructions with a kit (Glycospec; Abbott Laboratories, North Chicago, IL 60064) and an Abbott automated bichromatic analyzer (the ABA-100). An aliquot of each blood sample (drawn in EDTA-containing tubes) or standard was pipetted into the ABA sample cups. Ten microliters of each sample was automatically dispensed with 500 μL of hemolyzing reagent into the appropriate cuvette and the diff-
Table 1. Intra-Assay Coefficients of Variation for the Two Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean HbA1 (%)</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycospec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low concn</td>
<td>8.80</td>
<td>0.33</td>
<td>3.8</td>
</tr>
<tr>
<td>High concn</td>
<td>14.6</td>
<td>0.37</td>
<td>2.5</td>
</tr>
<tr>
<td>Bio-Rad</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low concn</td>
<td>6.75</td>
<td>0.17</td>
<td>2.5</td>
</tr>
<tr>
<td>High concn</td>
<td>16.1</td>
<td>0.34</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* 12 repeated HbA1 analyses on four different samples.

Table 2. Interassay Coefficients of Variation for Five Samples * by the Two Methods

<table>
<thead>
<tr>
<th>Glycospec</th>
<th></th>
<th>Bio-Rad</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean HbA1 (%)</td>
<td>SD</td>
<td>CV, %</td>
<td>Mean HbA1 (%)</td>
</tr>
<tr>
<td>9.1</td>
<td>0.47</td>
<td>5.2</td>
<td>5.3</td>
</tr>
<tr>
<td>10.9</td>
<td>0.54</td>
<td>5.0</td>
<td>6.6</td>
</tr>
<tr>
<td>13.7</td>
<td>0.72</td>
<td>5.2</td>
<td>10.1</td>
</tr>
<tr>
<td>19.9</td>
<td>1.18</td>
<td>8.0</td>
<td>14.2</td>
</tr>
<tr>
<td>20.6</td>
<td>0.85</td>
<td>4.1</td>
<td>15.1</td>
</tr>
</tbody>
</table>

* Samples analyzed daily for five consecutive days.

ference in absorbance at 560 and 633 nm was recorded. Fifty microliters of phytic acid reagent was dispensed into each cuvette and the difference was again recorded. For the absorbance absorption values to be accurate it was necessary to mix the reagents properly after each of the two dispensing sequences, by carefully shaking the multicuvette. The difference between the two recordings, corrected for total hemoglobin, was graphed against the percentage of HbA1 in the standards. The percentage of HbA1 in the blood samples could then be obtained from the graph.

Results

Precision

The within-assay precision of the two methods was determined for two concentrations of HbA1 (Table 1). The coefficient of variation (CV) was acceptable for both methods but was less with the Bio-Rad method, especially at low HbA1 values. The within-assay precision of the Glycospec method was further explored by analyzing 23 different blood samples in duplicate on one occasion. The mean HbA1 value was 14.4%, and by adopting a special formula the SD and CV were found to be 0.63% and 4.4%, respectively.

The between-assay CV for the two methods at five different HbA1 concentrations is shown in Table 2. The difference between the methods was small. The Bio-Rad method afforded slightly lower CV values.

HbA1 Values in Normal and Diabetic Subjects

In healthy subjects the mean (and SD) HbA1 value was 7.7 (1.3)% with the Glycospec and 6.8 (0.6)% with the Bio-Rad method. In the diabetic group the corresponding values were 14.0 (2.8)% and 11.0 (2.2)% respectively. Again, higher values were obtained with the Glycospec method, and the discrepancy was most pronounced in the diabetic group.

The correlation between the HbA1 values obtained with the two methods in the diabetic group was satisfactory ($r = 0.89$)

\[ SD = \sqrt{\frac{2(x_1 - x_2)^2}{2n}} \]

where $n$ is the number of samples.

Fig. 1. Correlation between HbA1 values obtained for blood from 122 diabetic subjects with the Glycospec and the Bio-Rad methods

as shown in Figure 1. The HbA1 values obtained with the Glycospec method were generally 2–4% higher than those obtained with the Bio-Rad method.

Effect of Storage and Anticoagulant Treatment

Storage of blood samples for several days affected the HbA1

Fig. 2. Effects of sample storage

Blood samples obtained from a normal subject (O—O) and a diabetic patient (■—■) were first stored at 4 °C for 4 days. On the 5th day, half of the samples were brought to room temperature (22 °C) while the other half was kept at 4 °C. Percent HbA1 was determined by both methods.
Table 3. Effect of Two Anticoagulants on HbA1c Values

<table>
<thead>
<tr>
<th>Assay method</th>
<th>HbA1c %  ± mean (SD)</th>
<th>EDTA</th>
<th>Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycospec</td>
<td>14.6 (0.37)</td>
<td>16.3 (0.33)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p&lt;0.001)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bio-Rad</td>
<td>11.1 (0.19)</td>
<td>10.8 (0.22)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(p&lt;0.01)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean of 10 analyses; 1 3.4 mmol/L; 4 14.3 int. units/mL.

values differently, depending on the assay used (Figure 2). Thus, the HbA1c concentrations in samples with initially low concentrations stored at 22 °C increased as determined with the Glycospec method; with the Bio-Rad method the increase was much less. Storage at 4 °C for at least 10 days did not significantly influence HbA1c values obtained by either of the two methods.

The effect of EDTA and heparin on the HbA1c values obtained by the two methods was also examined (Table 3). With the Glycospec method, heparin-treated samples yielded significantly higher HbA1c values; with the Bio-Rad method they were slightly lower.

Influence of 2,3-DPG on the HbA1c Values Obtained with the Spectrophotometric Method

Because phytic acid and 2,3-DPG bind to the same region of the hemoglobin molecule it was of interest to study the influence of exogenous 2,3-DPG on the spectrophotometric assay. Figure 3 illustrates the increase in HbA1c values when increasing amounts of 2,3-DPG were added to the blood samples before assay. A saturation was not evident, even at a concentration of added 2,3-DPG in the sample to be assayed as high as 23 mmol/L. A similar effect was obvious when 2,3-DPG was added to the three different HbA1c standards provided in the kit. The displacement of the standard curves after addition of 2,3-DPG is shown in Figure 4.

Discussion

The two methods displayed similar precision and reproducibility, although they represent different principles for the determination of HbA1c. The spectrophotometric method yielded consistently higher values than the microcolumn method, more obvious in diabetics than in normal subjects. Investigating possible reasons for this discrepancy, we found that 2,3-DPG caused a dose-related displacement of the standard curves of the spectrophotometric method. Furthermore, when 2,3-DPG was added to blood samples an approximately linear relationship was found between increase in percentage HbA1c and added amounts of 2,3-DPG. These results are consistent with a competition between 2,3-DPG and phytic acid for the same binding region in the hemoglobin molecule. Even at a 2,3-DPG concentration of 23 mmol/L (corresponding to 0.4 mmol/L in the final assay mixture) the percentage increase in HbA1c was nonsaturable. The concentration of phytic acid in the assay samples was 0.12 mmol/L, which means that phytic acid competes well with 2,3-DPG, even at a three- or fourfold excess of 2,3-DPG.

According to the manufacturer, the Glycospec HbA1c standards contain no 2,3-DPG (personal communication from Dr. E. G. Moore). In contrast, the concentration of 2,3-DPG in whole blood is about 2 mmol/L, as estimated from the 2,3-DPG content of erythrocytes reported by Ditzel and Standl (12). At this concentration of 2,3-DPG the standard curve yields 2–3% lower HbA1c values for a certain absorbance difference than those obtained from a standard curve with no added 2,3-DPG (cf. Figure 4). If the Glycospec values are corrected for this effect of 2,3-DPG, they then match those obtained by the Bio-Rad method.

Variations in the concentration of endogenous 2,3-DPG would not substantially influence the HbA1c values obtained with the spectrophotometric method. In the study of Ditzel and Standl (12) the range of 2,3-DPG (mean value ±2 SD) in whole blood was 1.6–2.8 mmol/L in diabetics. Even with the extreme values, the difference in HbA1c would only be about 0.5% according to Figure 3.

The Glycospec assay is sensitive to the anticoagulant used;
heparin-treated samples yield higher values than EDTA-treated samples. This is of importance in HbA1 analyses in children, because capillary-blood samples are usually drawn into heparinized tubes. Microcontainer tubes with EDTA (Becton Dickinson) are, however, available and should be used for capillary samples that are to be assayed by spectrophotometry.

Storage of blood samples was found to affect HbA1 values, depending on the assay used. Thus, after storage for a few days at 22 °C there was a sharp increase in HbA1 values determined with the Glycospec method, while the increase in HbA1 according to the Bio-Rad method was less. The reason for this discrepancy is obscure. Perhaps during storage the hemoglobin is partly denatured, a process that is enhanced at room temperature. This could lead to impaired binding of phytic acid and a diminished change in light absorption with a false increase in apparent HbA1. Results by the micro-column assay were only slightly affected by storage of the samples, which suggests that conformational changes of the hemoglobin molecule are of less importance for the chromatographic separation and elution of the glycohemoglobins.

The HbA1 fraction determined with the microcolumn method is heterogeneous. The spectrophotometric method seems to be more specific, because it measures only hemoglobin glycosylation at the N-terminal of the beta chains. However, compounds such as 2,3-DPG interfering at this region of the hemoglobin molecule might introduce an error. Furthermore, it is not known what extent the labile Schiff base of HbA1c is measured. The presence of hemoglobin F in the samples gives a false increase in the HbA1 value with both methods (10).

The spectrophotometric method offers several advantages as compared with the microcolumn method. It is less sensitive to small changes in temperature, pH, or ionic strength of the buffer. The method is easily automated and therefore well suited for large-scale analyses if problems with standards and calibration can be solved. It is easy to handle and no more prone to error than a routine analysis for blood glucose. It offers a unique possibility for the physician to rapidly monitor the long-term glucose status of his patients and to institute appropriate changes in the diabetes treatment regime without delay.

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References