Measurement of Hemoglobin A\textsubscript{1c} by a New Liquid-Chromatographic Assay: Methodology, Clinical Utility, and Relation to Glucose Tolerance Evaluated

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A chromatographic method for determining glycated hemoglobin (Hb A\textsubscript{1c}) by use of a new monodisperse cation-exchanger has been investigated. Hb A\textsubscript{1c} was separated from other "minor hemoglobins": Hb F, Hb A\textsubscript{2} (the glutathione adduct), and the acetaldehyde adduct in alcoholic solvents. The method was fully automated and a single column could be used for more than 1000 runs. The normal reference interval was 4.0–5.2%; the interval for diabetic outpatients was 5.6–12.4%. Within-run and the between-run CVs were <0.9% and 1.7%, respectively. Carbamylation in uremic patients who were undergoing hemodialysis increased the proportion of Hb A\textsubscript{1c} to 1%. Hb A\textsubscript{1c} results were compared with results from glucose tolerance tests. In our study, Hb A\textsubscript{1c} <5.5% excluded diabetes: subjects with Hb A\textsubscript{1c} >6.2% showed diabetes. If blood sampled during fasting had been screened with determinations of glucose and Hb A\textsubscript{1c} only 20% of referred subjects would have needed an oral glucose tolerance test for diagnosis of diabetes.

Additional Keyphrases: cutoff value \cdot uremia \cdot diabetes \cdot glycated hemoglobin \cdot chromatography, cation-exchange

Materials and Methods

Subjects

The control subjects were 63 ostensibly healthy adults (45 women), mean age 49 years (range 29–57 years), who had participated in a health-control survey.

To estimate the variation in the clinical setting, we determined Hb A\textsubscript{1c} in 41 consecutive patients visiting the Diabetic Clinic. Hb A\textsubscript{1c} was also determined for 20 nondiabetic alcoholic patients (drinking more than 300 g of ethanol per day) and 20 uremic nondiabetic patients undergoing hemodialysis.

The glucose tolerance test results were obtained from 137 subjects (67 of them nonpregnant women), mean age 48 years (range 18–82 years), who were referred to the Diabetic Clinic because of suspected diabetes.

Preparation of Samples

Blood was collected in EDTA-containing tubes or as capillary blood (100 μL) in heparinized tubes (Microtainer\textsuperscript{®}, Becton Dickinson, Rutherford, NJ). We mixed 0.6 mL of EDTA-treated blood with 10 mL of isotonic saline. After incubating the erythrocytes at 37 °C for 4 h to eliminate pre-Hb A\textsubscript{1c}, the labile intermediate form of Hb A\textsubscript{1c}, we sedimented them by centrifugation (5 min, 3000 × g) and discarded the supernatant fluid. Hemolysate was prepared by shaking the cells in a mixture of 2.5 mL of distilled water and 0.5 mL of CCl\textsubscript{4} for 30 s then centrifuging at 3000 × g for another 20 min. Before chromatography, we diluted the clear hemolysate ninefold with distilled water, which gave a hemoglobin concentration of approximately 3.5–5.0 g/L. Controls were prepared similarly, except we diluted concentrated hemolysate with an equal volume of a mixture containing, per liter, 50 mmol of KCN, 10 mmol of EDTA, and 200 mmol of Tris HCl (pH 8.0) before storing at −70 °C. KCN improves the stability during storage.

All Hb A\textsubscript{1c} determinations were performed as routine analyses at the Department of Clinical Chemistry at our hospital during the 18-month period.

Equipment

We used a "FPLC" (Fast Protein Liquid Chromatography) system from Pharmacia, Uppsala, Sweden, consisting of a controller (LCC-500), two P-500 pumps, and a UV-I single-path monitor equipped with an HR-10 flow cell and a 405-nm filter. The system had been automated for assay of either eight to 15 samples, by using one motor valve MV-7 and two motor valves MV-8, or 40 samples, by using the automatic injector ACT-100. An HP 3500 integrator (Hewlett Packard, Palo Alto, CA) was used for measuring the fractions.

Procedures

Chromatography with the Mono S HR 5/5 column. Hb A\textsubscript{1c} was separated from other hemoglobins by a complex LiCl-
gradient in 17 min, including regeneration. The start buffer (A) contained 10 mmol of sodium malonate and 0.2 g of sodium azide per liter (pH 5.7). Buffer B was buffer A plus 0.3 mol of lithium chloride per liter. Five liters of buffer could be kept in a refrigerator for at least one week. Before use, these buffers were degassed and filtered through a 0.45-μm pore-size filter. Flow rate was 2 mL/min, the monitor sensitivity range was 0.2 A full-scale, and the sample size injected was 50 μL. The gradient profile was as follows:

Volume, mL \[\text{Buffer} \ B\ \%,\ ]
\[\begin{array}{c|c}
0 & 0.2 \\
11 & 40 \\
15 & 50 \\
25 & 100 \\
28 & 100 \\
28 & 0.2 \\
34 & 0.2 \\
\end{array}\]

Titration curves were performed as described elsewhere (10).

The column and system (pumps) were stored filled with 0.2 g/L sodium azide solution. Once a week the whole system, excluding the Mono S column, was flushed with a 40 mL/L solution of sodium hypochlorite, followed by several changes of distilled water. After approximately 500 injections, or if the back pressure exceeded 4.0 MPa, the top filter assembly in the column was replaced. This ordinarily sufficed to reduce the pressure to 1.5–2.0 MPa again. To improve precision, we reviewed the total area recorded by the integrator for each sample, to ensure that the total hemoglobin concentration was within the range 3.5–5.0 g/L.

The prefiter 2 in the system (just before the column) was eliminated to improve the application of the 50 μL of diluted hemolysate onto the column. Capillary blood (minimum 100 μL) could also be used. Erythrocytes were incubated in saline at 37 °C for 4 h as earlier described. We then added 0.4 mL of distilled water and five drops of CCl₄ to prepare hemolysate from the washed erythrocyte pellet.

Glucose tolerance test. A 2-h oral glucose tolerance test was performed and interpreted according to WHO recommendations (11). Blood samples for analysis of glucose (whole blood, capillary) were taken 0, 30, 45, 60, 90, and 120 min after the intake of 75 g of glucose. Diabetes was diagnosed when blood glucose at 0 min was >6.7 mmol/L or at 120 min was >11.1 mmol/L. Impaired glucose tolerance was diagnosed when glucose at 0 min was <6.7 mmol/L and at 120 min was 7.8–11.0 mmol/L. Blood glucose was assayed by a hexokinase method. To correlate results by each test, we also collected at 0 min a blood sample for assay of Hb A₁c.

Statistical analysis. The Mann–Whitney U-test and the χ² test were used for statistical evaluation. All tests were two-tailed and p < 0.05 was considered significant. The results are presented as mean ± SD.

Results

Development of the Method

To ascertain ideal chromatographic conditions, we prepared an electrophoretic titration curve from a hemolysate containing increased amounts of Hb A₁c. The most pronounced difference in electrophoretic mobility and net charge was at about pH 5.7 in the cationic side (Figure 1). Because the differences in net charge between hemoglobin fractions are small, a complex salt gradient was formed. Figure 2 (top) shows the most common pattern when Hb F and Hb A₃ are present as interfering substances. Our aim was to produce well-defined peaks so the integrator could calculate reproducible areas. Theoretically, the whole program (including regeneration) can be run in 7 min (14 mL); however, we obtain higher resolution with a 17-min gradient (34 mL), which is advisable in the presence of interferences such as Hb F or A₃ (Figure 2 (bottom)). This gradient also separates most of the genetic variants of hemoglobin. By practicing careful maintenance of the Mono S column, ultralitering all the buffers, including a bacteriostatic agent (sodium azide), and replacing the top fitting periodically, we could use one Mono S column for more than 1000 runs.

Temperature

To determine the effect of temperature, we chromatographed samples at five different temperatures from 17.5 to 30.0 °C. Results obtained within the range 17.5–27.5 °C were identical. A slight increase (+0.4%) of Hb A₁c was obvious at 30 °C. Therefore, we operated the system at normal room temperature (22–25 °C).

Storage and Preparation of Samples

Blood specimens could be stored as EDTA-blood for as long as seven days at 4 °C without loss of Hb A₁c. Concentrated hemolysate (approximately 30 g of hemoglobin per liter) could be stored as long as three days at 4 °C. Diluted hemolysates were stable for 12 h at room temperature. Controls stabilized in KCN–EDTA–Tris HCl buffer could be stored at −70 °C for at least 12 months.

Assay Precision

The within-assay precision of the method was determined by analyzing samples from two patients having different
proportions of Hb A$_{1c}$ (Table 1). The between-assay precision was calculated from results obtained for 50 frozen quality-control samples during six months.

**Clinical Utility and Correlation with Oral Glucose Tolerance Test**

In the reference samples (Figure 3) collected from 63 healthy subjects and analyzed during two months, mean Hb A$_{1c}$ was 4.6% (SD 0.3%, range 3.9–5.3%). In 41 consecutive diabetic patients visiting the diabetic clinic, the mean Hb A$_{1c}$ was 8.6% (SD 1.6%, range 5.6–12.4%); in nondiabetic alcoholics the respective values were 4.8%, 0.4%, and 4.0–5.6%, and in uremic nondiabetic subjects 5.7%, 0.5%, and 4.9–6.8%. In glucose-tolerance-tested subjects (Figure 3), the results (mean ± SD, and range) were 4.9 ± 0.5% (3.7–6.0%) in 66 subjects with a normal result for the oral glucose tolerance test; 5.3 ± 0.4% (4.5–6.1%) in 29 subjects with impaired glucose tolerance; and 6.6 ± 1.1% (5.5–11.2%) in 42 subjects with a diabetic result for the oral glucose tolerance test. Mean Hb A$_{1c}$ for the subjects with impaired glucose tolerance was significantly higher (p < 0.01) than for subjects with a normal oral test result, and significantly lower (p < 0.001) than for subjects with a diabetic oral test result. From the results shown in Figure 3, we interpreted Hb A$_{1c}$ <4.5% as indicating a normal glucose tolerance; 4.5–5.4%, possible impaired glucose tolerance, 5.5–6.1%, possible impaired glucose tolerance or diabetes; and >6.2%, diabetes. The sum of the glucose values obtained during oral glucose tolerance test in the 13 subjects with a normal oral glucose test and an Hb A$_{1c}$ value of 5.5–6.1% was significantly higher (p < 0.001) than in 15 subjects with a normal oral glucose tolerance and Hb A$_{1c}$ <4.5%: 51.8 ± 8.8 and 40.2 ± 5.7 mmol/L, respectively. The mean fasting glucose did not differ significantly between 13 subjects with a normal glucose tolerance test result and an Hb A$_{1c}$ of 5.5–6.1% and the nine patients with impaired glucose tolerance and the same Hb A$_{1c}$ 5.2 ± 0.6 and 5.4 ± 1.0 mol/L, respectively.

Figure 4 shows that determining Hb A$_{1c}$ and blood glucose in single samples of blood collected from the fasting subject reduced considerably the need for a followup 2-h oral glucose tolerance test. Only 28 of 137 (20%) subjects would have needed a 2-h oral glucose tolerance test to diagnose or rule out diabetes if such a screening test had been used. If only a fasting blood glucose had been measured, but not Hb A$_{1c}$, 87 of 137 (64%) subjects would have needed an oral glucose tolerance test, a significant (p < 0.001) increase.

**Discussion**

**Methodological Considerations**

Titration curves are an ideal tool in protein chemistry for dealing with complicated separations (10). The general pattern we obtained for Hb A$_{1c}$ indicated the use of a cationic resin or chromatofocusing as the methods of choice. Tests within the pH range of 5.5–6.0 showed that pH 5.7 gave optimal separation.

Several promising HPLC systems have recently been described (7–9), all superior to the original Bio-Rex 70 (12). The system we used was introduced in a congress report in 1983 (13). Here we present our experience after 10 000 automated runs performed during 18 months in our routine laboratory. The most interesting point was the precision and accuracy of the method. The system was easily operated.

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**Table 1. Precision of the Assay**

<table>
<thead>
<tr>
<th>Hb A$_{1c}$ %</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>4.13</td>
<td>0.019</td>
<td>0.47</td>
</tr>
<tr>
<td>Diabetic</td>
<td>20</td>
<td>7.85</td>
<td>0.074</td>
<td>0.94</td>
</tr>
<tr>
<td>Between assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>50</td>
<td>4.91</td>
<td>0.082</td>
<td>1.68</td>
</tr>
</tbody>
</table>
Because the built-in integrator was dependent on knowing the exact amount of hemoglobin applied, which had to be
determined individually for each sample, we added an
external integrator to the system. The monodisperse cation
resin, ‘Mono S,’ does not shrink or swell, or leak functional
groups. The large numbers of hemoglobin samples that
could be run on a single Mono S column kept equipment
costs low.

In normal erythrocytes 0.3% of the hemoglobin is present
as a labile precursor, pre-Hb A1c, in which glucose is
attached to the N-terminal amino acid of the β-chain by a
reversible Schiff-base reaction. The pre-Hb A1c may increase
to 3.5–4.0%; because the precursor mirrors the actual glu-
cose concentration at the time of the blood sampling (14),
this disturbs the evaluation of long-term metabolic control
measured via Hb A1c. Pre-Hb A1c can be partly separated
from Hb A1c in ion-exchange chromatography by modifying
the gradient. However, we prefer pre-incubation of the
erthrocytes for 4 h at 37 °C in saline to eliminate the pre-
Hb A1c (15). Earlier tests of the pre-incubation procedure by
isoelectric focusing confirm that no methemoglobin is pro-
duced (14). Faster methods are available for eliminating
pre-Hb A1c, but this procedure fits well with the working
plan in our laboratory. All samples (40 per day) can be
prepared during the day and assayed by the automatic
FPLC-system during the night.

Hemoglobinopathies often interfere with Hb A1c results
(Figure 5), but our system detects most subjects with
hemoglobinopathies. So far, we have found four variants
with double A and A1c peaks, some of which we have
investigated for specific amino acid substitutions, using the
same equipment system. We separated α- and β-chains by
reversed-phase chromatography on a C18/C8 silica column;
for tryptic peptides we used a C2/C18 column (16). The LiCl
gradient can easily be modified to be more suitable for
separating abnormal hemoglobins; however, we prefer iso-
electric focusing to characterize patients with suspected
abnormal hemoglobins.

Clinical Considerations

The natural skepticism about the clinical utility of glyca-
ed hemoglobin measurements has been related to problems
with assay precision and reproducibility (4–6). The narrow
reference interval (4.0–5.2%) in our method indicates a high
precision. The clinical utility of our method is further
strenthened by the high reproducibility shown in the
clinical setting. Patients in stable metabolic control who
were monitored prospectively showed only low variations in
Hb A1c; in other patients, Hb A1c declined smoothly when
metabolic control was improved.

The specificity of the method, as shown by our detection of

**Fig. 4.** Effects of different screening procedures on the need for an oral glucose tolerance test (OGTT) to diagnose 137 subjects referred because of suspected diabetes (DM); (left) Hb A1c and fasting blood glucose (FB-glucose) were determined before the oral glucose tolerance test; (right) only fasting blood glucose was determined before the oral glucose tolerance test.

Larger numerals in each box indicate the number of subjects, smaller numerals indicate the cutoff values used for the tests listed in the center column.
abnormal hemoglobin variants in some patients, reduces the risk for false Hb A1c values in diabetic subjects with hemoglobin variants. The acetaldehyde adduct in the hemoglobin of alcoholics may theoretically interfere with Hb A1c determinations. However, even though we have not been able to separate the acetaldehyde fraction (17), we found no interference in alcoholics, their Hb A1c range corresponding to our reference values. In uremic patients, on the other hand, carbamylation increased Hb A1c values by as much as 1%. This must be considered in evaluating Hb A1c results for diabetic patients with uremia.

Correlation with the Oral Glucose Tolerance Test

The increasing number of subjects with suspected diabetes being referred to our Diabetes Care Unit for diagnosis has increased the demand for time-consuming oral glucose tolerance tests. A screening method reducing the need for an oral glucose tolerance test would be convenient, and we tested our new automated Hb A1c assay for this. Mean Hb A1c values differed for all three WHO-defined groups: normal, impaired glucose tolerant, and diabetic. A similar differentiation by Hb A1c has been shown with cation-exchange chromatography (18, 19), affinity chromatography (20, 21), and electrophoretic endomosis (22, 23), indicating that Hb A1c values might be useful in diagnosing diabetes or establishing impaired glucose tolerance. However, individual Hb A1c values determined by cation-exchange chromatography overlapped for the three who groups (18). Another study reported that affinity chromatography results had less overlapping, although the impaired glucose tolerance group was small (n = 5) (20). The cutoff values we used [Hb A1c < 5.5% excluded diabetes, > 6.2% confirmed diabetes], combined with results for a fasting blood glucose test, substantially reduced the need for a 2-hour oral glucose tolerance test to diagnose diabetes. However, subjects with impaired glucose tolerance could not be discriminated; an oral glucose tolerance was still required, to establish impaired glucose tolerance. A similar difficulty in defining subjects with impaired glucose tolerance by their Hb A1c determinations has been reported by others (21). The wish to detect diabetes in an asymptomatic subject rests on the assumption that diabetic treatment is indicated (11). On the other hand, treatment of impaired glucose tolerance has not been clearly demonstrated to prevent vascular complications or the development of overt diabetes (24).

An interesting observation was that the Hb A1c range in patients referred for an oral glucose tolerance test was wider than that for healthy control subjects. The patients who were referred because of suspected diabetes may have subtle carbohydrate intolerance not detected by results for the oral glucose tolerance test but leading to Hb A1c values exceeding the range of values from healthy subjects. Finally, subjects with normal glucose tolerance but with Hb A1c 5.5–6.1% showed higher glucose values during the oral glucose tolerance test than did those with a normal glucose tolerance and lower Hb A1c values.

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References