intravenous administration to a healthy man.

Although methylprednisolone possesses pharmacological properties similar to those of dexamethasone, it is unlikely that these two agents would deliberately be used in combination in treating a patient. Therefore, we believe it is a good choice for use as an internal standard. The chromatographic procedure described differs from those previously reported by the use of a fast, efficient, and inexpensive solid-phase extraction step. The process takes approximately 40 min to the completion of the drying step, so that about 20 samples of plasma can be analyzed within an 8-h work day. We believe this procedure is suitable for clinical applications and offers significant advantages over previously described methods.

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References


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An Automated Liquid-Chromatographic System for Convenient Determination of Glycated Hemoglobin A\textsubscript{1c}

Ahmed K. Saleh\textsuperscript{1} and Mohamed A. A. Moussa\textsuperscript{2}

For quantification of glycated hemoglobin (HbA\textsubscript{1c}), we compared an automated system (Pharmacia Fast Protein Liquid Chromatography), which separates and determines HbA\textsubscript{1c} with a commercial disposable-minicolumn kit (Boehringer-Mannheim), which separates total HbA\textsubscript{1} and we studied 41 diabetic women and 79 apparently normal women on their first postpartum day. The automated method was more precise (within-run CV 0.98–4.16%) than the kit method (within-run CV 3.67–7.77%). Results by the two methods correlated well for both control (p < 0.001) and diabetic (p < 0.05) groups. Values for HbA\textsubscript{1c} correlated significantly with fasting blood-glucose concentrations in controls and diabetics (intraclass correlation coefficient \( r = 0.822 \) and 0.851, respectively, \( p < 0.001 \)) as well as with 1- and 2-h values for glucose after a 75-g glucose load in the control group (\( r = 0.649 \) and 0.846, respectively, \( p < 0.001 \)). For HbA\textsubscript{1} no such significant correlation was found except with values for fasting blood glucose in diabetics (\( r = 0.745, p < 0.001 \)).

Evaluation of HbA\textsubscript{1c} was a more sensitive index of glycemic status. The automated system is convenient, reliable, and easily operated.

Additional Keyphrases: diabetes • glucose tolerance test • pregnancy • fetal hemoglobin (HbF)

Minor variants of adult hemoglobin (HbA\textsubscript{0}) have been identified as hemoglobin A\textsubscript{1a}, A\textsubscript{1b}, and A\textsubscript{1c} according to their order of elution on cation-exchange chromatography (1). Hemoglobin A\textsubscript{1c} (HbA\textsubscript{1c}) is similar to HbA\textsubscript{0} except that glucose is linked to the amino-terminal valine of the \( \beta \)-chain by means of a Schiff base (2). The Schiff base adduct slowly undergoes the Amadori rearrangement to stable ketoamine (3–5). "Glycated hemoglobin" is the term recently applied to describe this nonenzymatic linkage (7). The N-terminal valine is not the only glycation site of HbA\textsubscript{0}. Ketoamine linkages have also been found with \( \varepsilon \)-amino groups of lysine residues in both \( \alpha \) - and \( \beta \)-chains of HbA\textsubscript{0} (8). However, glycation of N-terminal valine causes the molecule to undergo a change in its isoelectric point, which allows it to be separated from other hemoglobins. On the other hand, hemoglobin A\textsubscript{1a} has been further separated into two components, A\textsubscript{1a1} and A\textsubscript{1a2}, which co-chromatograph with hemo-
globin adducts of fructose 1,6-diphosphate and glucose 6-phosphate (9). Hemoglobin A1b is nonglycated and is probably a deamidated product of HbA0 (10).

Rahbar, in 1968, drew attention to the increased amounts of the "fast-moving" hemoglobin—as measured by electrophoresis—in diabetic patients (11); this was found to be similar to HbA1c. Neither A1a1 nor A1a2 is increased in diabetes. The concentration of HbA1c is proportional to the blood glucose concentration to which the erythrocyte has been exposed during its life span (3). Hence, there is an increasing clinical demand for quantification of HbA1c because it reflects the long-term control of diabetes (12), and probably for retrospective diagnosis of gestational diabetes (13, 14).

Glycated hemoglobin has been determined by several techniques, most of them laborious, time-consuming, and sophisticated. The Fast Protein Liquid Chromatography (FPLC) automated system has been designed by Pharmacia Fine Chemicals, Upsala, Sweden, for separating proteins—including HbA1c—on "high-performance" columns packed with uniform, monodisperse spheres so as to allow rapid identification of proteins or assessment of microheterogeneity of single proteins, at specific pH and flow characteristics and with moderately increased pressure. Some applications of the system have been reported in the archival literature (15–17) but, to our knowledge, not yet regarding the determination of HbA1c.

The purpose of this work is to illustrate the application of this automated system in the separation of HbA1c. The resolution is based on the work of Jeppsson et al. (18). We compared results with those by one of the disposable minicolumn kits with which HbA1 is determined.

Materials and Methods

Subjects

Blood was sampled from 41 insulin-treated diabetic women and 79 apparently normal women, as controls, on their first postpartum day. Potentially diabetic women (women who had a family history of diabetes or a poor obstetric history or who had ever given birth to babies weighing ≥4.5 kg) were excluded from the control group. None of the patients showed any abnormal hemoglobin variant in their blood. The relevant clinical data about both groups are shown in Table 1. The tests for HbA1c were carried out to check the glycemic status of the diabetic patients. Values from the controls were used to determine our normal reference interval. From each woman, who had fasted for 10 h, we collected 2 mL of venous blood in EDTA-containing tubes, 1 mL for each method. To estimate concentrations of plasma glucose, we collected 2 mL of venous blood in tubes containing fluoride–oxalate. These specimens were kept at 4 °C for 1–2 h, then transferred refrigerated to the laboratory.

Procedure for Automated Chromatography

Hemolysate preparation. Erythrocytes from 1 mL of the venous blood, well mixed with EDTA, were washed three times with physiological saline, centrifuged (10 min, 3000 rpm), and the supernates discarded. To eliminate the labile glycated hemoglobin fraction, we added 2 mL of the saline to the washed erythrocytes, mixed well, and left the mixture overnight at room temperature (19). We lysed the packed cells by adding 0.5 mL of distilled water and 200 μL of CCl4. Debris was removed by centrifugation (15 min, 3000 rpm) and the very clear supernatant fluid was pipetted into a disposable reaction vial (L.I.P. Equipment and Services Ltd., West Yorkshire, U.K.), then stored at −80 °C for not longer than four weeks. For analysis, we diluted the hemolysate with five volumes of sodium malonate (10 mmol/L, pH 5.7) and filtered it through a 0.22-μm pore-size disposable filter (Millipore Corp., Bedford, U.K.). Eight to 15 samples, each in a 2-mL glass tube, could be fixed in the sample holder to be analyzed simultaneously. All the glassware used was cleaned thoroughly and stored dry in an oven.

Buffers and gradients. Buffers were prepared freshly every three days, and were stored at 4 °C when not in use. The start buffer (Buffer A) for use with the cation-exchange column was the above-mentioned malonate buffer. The mixture used to effect a salt gradient (Buffer B) was Buffer A plus 0.3 mol of lithium chloride per liter. Before use, these buffers were degassed and filtered through 0.45-μm pore-size filters. The elution gradient was produced by pumps according to the computed method in the system integrator in the following concentrations:

<table>
<thead>
<tr>
<th>Vol, mL</th>
<th>% of Buffer B in eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>40.0</td>
</tr>
<tr>
<td>11</td>
<td>50.0</td>
</tr>
<tr>
<td>14</td>
<td>100.0</td>
</tr>
<tr>
<td>17</td>
<td>100.0</td>
</tr>
<tr>
<td>18</td>
<td>0.2</td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Chromatography. The automated chromatographic system used in this study was manufactured by Pharmacia (Figure 1). Only 50 μL of the diluted hemolysate was loaded onto the column and eluted in 16 mL of the starting Buffer A. The flow rate of the buffers during the elution was 2 mL/min. Chromatography was performed at room temperature. Separation of HbA1c and regeneration of the column was complete in 10 min.

Electrophoresis of the glycated hemoglobin fraction was performed on microcolumns packed with cation-exchange resin (Boehringer Mannheim GmbH, Diagnostica, Mannheim, F.R.G.), according to the manufacturer's instructions. A jacket of incubating water around the columns was thermostatized at 23 °C. Absorbances of the collected eluates were measured at 415 nm with a spectrophotometer (Uvidec-40; Jasco Medical Instruments, Inc., Tokyo 113, Japan).

Estimation of Plasma Glucose

We determined the concentration of glucose in plasma by the glucose oxidase procedure (20). Fasting blood glucose

Table 1. Basic Characteristics of the Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n = 79)</th>
<th>Diabetics (n = 41)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5th</td>
<td>95th</td>
</tr>
<tr>
<td>Patient's age, yr</td>
<td>25</td>
<td>38</td>
</tr>
<tr>
<td>Patient's wt, kg</td>
<td>55</td>
<td>78</td>
</tr>
<tr>
<td>Patient's parity</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>6.38</td>
<td>8.56</td>
</tr>
<tr>
<td>HbA1, %</td>
<td>6.19</td>
<td>9.56</td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
<td>4.50</td>
<td>5.28</td>
</tr>
<tr>
<td>after fasting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 h after glucose load</td>
<td>7.61</td>
<td>11.22</td>
</tr>
<tr>
<td>2 h after glucose load</td>
<td>5.44</td>
<td>9.56</td>
</tr>
</tbody>
</table>

*No. of previous births. **Not done for diabetics.
Statistical Methods

We tested the normality of the studied variables by fitting the data to the normal distribution by the chi-squared test, and by looking into the skewness and kurtosis. We decided that nonparametric statistics (21, 22) were appropriate, and used the Mann-Whitney test to assess the significance of the difference between two groups. We used the intraclass correlation coefficient (r) to assess the strength of association between two variables (23). The precision of each method was assessed by calculating the coefficient of variation.

Results

Monitoring the hemolysate elements at 405 nm produced three main peaks (Figure 2), identified by their retention times on the automated analyzer as fetal hemoglobin (HbF) at 5 mL, HbA1c at 7 mL, and HbA0 at 14 mL (Figure 3); methemoglobin is eluted as a shoulder on the HbA0 peak. HbA1a and HbA1b peaks were eluted before HbF. HbA1c was calculated by the system as the percentage its peak area was of the total areas under the peaks. The means of 10 determinations of HbA1c by the automated system were compared with 10 results for HbA1c determined by the disposable kits in four samples (Table 2). The automated system was more precise (within-run CV 0.98–4.16%) than the kits (within-run CV 3.67–7.77%). Values of HbA1c, HbA1a, and blood glucose concentrations for the control and diabetic patients are shown in Table 1. HbA1c and HbA1a values were higher in diabetics than in the controls. Values for HbA1c correlated significantly with those for HbA1a in controls (r = 0.518, p < 0.001) and the diabetics (r = 0.392, p < 0.05). HbA1c correlated well with the concentrations of plasma glucose measured in the controls (r = 0.822 for fasting blood glucose, r = 0.849 for 1-h blood glucose, r = 0.846 for 2-h blood glucose, p < 0.001) as well as in the diabetics (r = 0.851, p < 0.001 for fasting blood glucose). Scatter diagrams (Figure 4) illustrate the correlations of HbA1c with concentrations of plasma glucose in both groups. HbA1c showed no such significant correlation except with values for fasting blood glucose in diabetics (r = 0.745, p < 0.001).

Discussion

Several methods for quantification of glycated hemoglobin exist, but the ideal method for routine clinical use is not yet available. Conventional chromatographic techniques are based on cation-exchange resins. Early methods involv-
ing long columns required several days to run and regenerate the column (1). With the development of short and minicolumn methods, the procedure became faster (24–27). With the automated system used in this study, the time of running and re-equilibrating the column has been decreased to 10 min. This can facilitate the large scale clinical investigations necessary to define the role of HbA1c in diagnosis and monitoring the control of diabetes.

We confirmed that neither HbF—which might be encountered because of fetomaternal transfusion during delivery—nor HbA1b nor HbA1c affects the HbA1c peak resolved by this automated system. The time required to eliminate interference of the labile pre-HbA1c can be shortened by incubating the erythrocytes at 37 °C for a few hours (28). To overcome the problem of increased column resistance to flow encountered in “high-pressure” chromatographic techniques (25), the automated system uses a short column (Mono S) prepacked with uniform, rigid 10-μm monodisperse spheres. However, it is necessary to wash the column regular every five runs to remove residual contaminants that have built up; this avoids excessive back pressure and increases the life span of the column. At a flow rate of 2 mL/min the column operated at a back pressure of 2070 kPa.

We find that the Pharmacia automated system is convenient, reliable, and easily operated. Its results for HbA1c are precise (within-run CV <5%) and correlate with concentrations of plasma glucose in both control and diabetic subjects. We conclude that it is useful in assessing the glycermic status of patients.

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Fig. 4. Scatter diagrams of HbA1c vs the concentrations of blood glucose in the control and diabetic groups. FBG, blood glucose after fasting; 1hBG, blood glucose 1 h after glucose load; 2hBG, blood glucose 2 h after glucose load.

References
Determination of Dehydroepiandrosterone Sulfate in Plasma by a One-Step Enzyme Immunoassay with a Microtitre Plate

Tarun Kumar Dhar,¹ Christian Müller, and Manfred Schöneshöfer²

We have developed a rapid and cost-effective enzyme immunoassay for dehydroepiandrosterone sulfate (DHEA-S) in plasma, performed with samples on a microtitre plate within 2.5 h. No extraction or centrifugation steps are involved. The 3-hemisuccinate of dehydroepiandrosterone is labeled with horseradish peroxidase, then mixed with hydrogen peroxide substrate in the presence of the chromogen, tetramethylbenzidine. The detection limit of the assay is 12.5 pg of DHEA-S per well. Intra- and interassay CVs at three steroid concentrations (12.8, 1.28, and 0.16 μmol/L) ranged from 2.3 to 5.4% and 6.1 to 8.4%, respectively. Results correlated well (r = 0.95) with those of a radioimmunoassay with iodinated DHEA-S. The turnaround time for 41 samples (in duplicate) is 2.5 h, which includes 2 h of incubation time. The sensitivity of this one-step version and the linearity of its standard curve are equivalent to those of a less practicable two-step version. This technique may replace coated-tube enzyme immunoassays for routine use.

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Additional Keyphrases: reference interval · steroids · RIA compared · pituitary/adrenal disorders · pediatric chemistry

Dehydroepiandrosterone sulfate (DHEA-S)² is secreted by the zona reticularis of the adrenal cortex. Because of its long biological half-life and little diurnal variation, this steroid serves as an effective biochemical marker for adrenal production of androgens (I, 2). It is also useful in the biochemical differential diagnosis of the etiology of Cushing's syndrome (3). Radioimmunoassays (RIAs) are the most widely used techniques for determining DHEA-S (4), nonisotopic immunoassays of DHEA-S having so far been limited to a chemiluminescence enzyme immunoassay (5). Although heterogeneous enzyme immunoassays are more sensitive than homogeneous assays for determining low-Mᵦ compounds, the tedious endpoint determination of enzyme activity in the widely used "solid-phase" techniques makes them impracticable. Here, we describe a nonisotopic immunoassay of DHEA-S in plasma, based on a competitive enzyme immunoassay with microtitre plate (MP-EIA). In addition to the analytical qualities of heterogeneous EIAs, this technique also offers advantages of cost and practicability.

¹ Nonstandard abbreviations: DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DHEA-HS, dehydroepiandrosterone hemisuccinate; EIA, enzyme immunoassay; MP-EIA, microtitre plate–enzyme immunoassay.