Evaluation of Three Minicolumn Procedures for Measuring Hemoglobin A₁
Glenn T. Hammons,¹ Kurt Junger, Jay M. McDonald, and Jack H. Ladenson²

We have evaluated three commercially available column-chromatographic methods (Isolab, Helena, and Bio-Rad) for the determination of "fast" hemoglobin (HbA₁). All three methods correlated with HbA₁c measurements by "high-performance" liquid chromatography for 121 samples from diabetic patients, with the Isolab method showing the highest correlation (r = 0.921). The Isolab and Helena methods gave results that were linear with proportions of HbA₁ as great as 30%; results by the Bio-Rad method were slightly nonlinear at values > 15%. The Isolab method showed better within- and between-assay precision (CV) than the other two methods and was considered the simplest to perform by each of four different technologists. We recommend use of the Isolab method over the other two tested and believe that this procedure will be valuable for monitoring long-term glycemic control in diabetic patients.

Additional Keyphrases: chromatography, column • "kit" methods • glycohemoglobin • diabetes

Most clinical and analytical studies of glycosylated hemoglobin have been based on measurement of HbA₁c, the largest component of the "fast" hemoglobins. However, a recent study from our laboratory (1) has established that, for clinical use, the measurement of HbA₁ is equivalent to measurement of HbA₁c. Because the methods for measurement of HbA₁c are generally not well suited to the routine clinical laboratory, simple and more rapid methods have been developed to measure HbA₁. We have evaluated three of these commercially available techniques, which are based on cation-exchange chromatography, and have compared results by each of them with HbA₁c as measured by "high-performance" liquid chromatography (HPLC) for 121 samples from diabetic patients.

Materials and Methods

We determined HbA₁ by three commercial procedures, all of which involve cation-exchange chromatography in "minicolumns": Fast Hb Test System (Isolab Inc., Akron, OH 44321), Hemoglobin A₁ by Column (Bio-Rad Laboratories Inc., Richmond, CA 94804), and Glycosylated Fast Fraction, Hemoglobin Quik Column (Helena Laboratories, Inc., Beaumont, TX 77704). All procedures were performed according to manufacturers' instructions. We measured HbA₁c by the HPLC technique of Davis et al. (2). We also determined HbA₁c by the HPLC procedure with the addition of HbA₁c and HbA₁c (1). To assess method precision, we analyzed multiple aliquots of a single patient's hemolysate, a hemolysate prepared from a pool of patients' samples, and commercially produced quality-control materials purchased from Isolab, Inc. (Fast Hemoglobin Control), Helena Laboratories, Inc. (Lyophilized G-Hb Quik Column Control), and ClinTech Diagnostics Corp., Mt. Sinai, NY 11766 (GlycoTrol). The hemolysates from patients' samples were prepared by pooling erythrocytes, washing the cells with isotonic saline (9.0 g/L), and lysing the cells with three volumes of lysing solution (0.15 g of NaNO₃ and 1.0 g of ethyl hexadecyl dimethyl ammonium bromide per liter). This hemolysate was then dialyzed for 24 h against phosphate buffer (per liter, 4.59 g of NaH₂PO₄ · H₂O, 2.23 g of Na₂HPO₄ · 2 H₂O, and 0.65 g of KCl, pH 6.67, at 24 °C) and frozen in small aliquots at −70 °C. The HbA₁c values of these frozen hemolysates were stable for at least two months. Within-assay precision was determined by measuring the various materials 10 or 20 times in succession; between-assay precision was determined by analyzing the various quality-control materials once in each of several separate assays. Only one assay by each method was performed per day.

To assess the linearity of the various HbA₁ procedures, we prepared a hemolysate from pooled fresh patients' samples and fractionated it by column chromatography according to the method of Trivelli et al. (3). The eluted fractions were pooled into a "fast" fraction (HbA₁a + HbA₁b + HbA₁c) and a "slow" fraction, and these two pooled fractions were dialyzed against phosphate buffer (see above) and concentrated by ultrafiltration. The fractions containing "slow" and "fast" hemoglobin were then mixed to give test samples containing 3–30% fast hemoglobin and analyzed in duplicate or triplicate by each method.

For assay comparison studies, we obtained from normal volunteers and from pediatric and adult diabetic patients blood anticoagulated with EDTA or heparin. The samples were analyzed immediately or stored in the refrigerator as whole blood or packed erythrocytes until assays were run (within three days). For each sample, all the minicolumn procedures were performed on the same day. The HPLC analysis was completed on the same day for most samples, a day later for a few.

The stability of the values for HbA₁ (Isolab) and HbA₁c was assessed by using freshly drawn samples anticoagulated with EDTA from three patients with diabetes mellitus (HbA₁ values between 10 and 13%). Samples were assayed in triplicate 2–6 h after collection, then stored at 4 or 24 °C as whole blood and assayed again at periodic intervals. The results at the various times were compared with the initial value by Student's paired t-test.

Results

Precision. The within-assay precision for the three minicolumn methods with various quality-control materials or patients' samples is shown in Table 1. The best precision was obtained with the Isolab procedure, regardless of the type of sample tested.

The between-assay precision, for both commercial quality-control materials and patients' frozen hemolysates, is shown in Table 2. Again, the best precision was obtained with the Isolab method. In two instances (Isolab QC No. 1 with the Bio-Rad method and Isolab QC No. 2 with the Helena method), the CVs were unusually large, apparently because of
changes in the particular lot of the Bio-Rad or Helena columns used: the precision within each lot was substantially better than the precision across all lots used for these quality-control materials.

Linearity. The values obtained for fast hemoglobin fractions of 3–30% prepared by the technique of Trivelli et al. (3) are shown in Figure 1. The Isolab and Helena procedures gave apparently linear results over this range, but the Bio-Rad procedure appeared nonlinear for samples in which the HbA1c fraction was greater than 15%.

Accuracy. Samples for 121 diabetic patients with various degrees of glycemic control were assayed by all three minicolumn procedures and by HPLC (HbA1c). The data were obtained over a 10-month period by four different technologists, with use of columns of several lot numbers (Figure 2). HbA1c values obtained by all three methods were highly correlated with HbA1c by the HPLC technique, the Isolab technique having the highest correlation (r = 0.921). By comparison, the HbA1c results by HPLC (data not shown) had a correlation coefficient of 0.979 (HbA1c = 1.08 HbA1c + 2.80) for the same samples. This relationship was similar to that reported previously (1).

Sample stability. Hemoglobin A1 and HbA1c values showed no change for at least 48 h when EDTA-anticoagulated whole blood was stored at 4 °C and for at least 24 h when it was stored at 24 °C.

Discussion

With these three presumably similar commercially available methods for measuring HbA1c by cation-exchange chromatography, the absolute values obtained can vary. All the methods correlated well with HbA1c values, the Isolab procedure showing the best correlation (r = 0.921). The slope of the least-squares regression line was less than one in all cases, with Bio-Rad (0.87) and Isolab (0.79) being closest to one. The Isolab and Helena methods gave linear responses up to 30% fast hemoglobin, but the Bio-Rad procedure appeared slightly nonlinear at the higher values tested. The Isolab method gave the best precision both intra- and inter-assay.

Considering all the data, we recommend the Isolab method. It had the best precision and agreement with HbA1c and was judged simplest to perform by the four technologists who used

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**Table 1. Within-Assay Precision of Hemoglobin A1 Methods**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Isolab</th>
<th>Bio-Rad</th>
<th>Helena</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (and SD), %</td>
<td>CV, %</td>
<td>Mean (and SD), %</td>
</tr>
<tr>
<td>Isolab QC</td>
<td>5.8(0.05)</td>
<td>0.9</td>
<td>5.3(0.19)</td>
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<tr>
<td>Helena QC</td>
<td>9.7(0.10)</td>
<td>1.0</td>
<td>9.4(0.19)</td>
</tr>
<tr>
<td>Patient pool</td>
<td>11.4(0.10)</td>
<td>0.9</td>
<td>10.0(0.18)</td>
</tr>
<tr>
<td>Hemolysate no. 1</td>
<td>9.0(0.26)</td>
<td>2.9</td>
<td>8.6(0.56)</td>
</tr>
<tr>
<td>Hemolysate no. 2</td>
<td>7.8(0.13)</td>
<td>1.7</td>
<td>9.3(0.80)</td>
</tr>
<tr>
<td>Hemolysate no. 3</td>
<td>12.3(0.37)</td>
<td>3.0</td>
<td>13.1(0.50)</td>
</tr>
</tbody>
</table>

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**Table 2. Between-Assay Precision of Hemoglobin A1 Methods**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Isolab</th>
<th>Bio-Rad</th>
<th>Helena</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (and SD), %</td>
<td>CV, %</td>
<td>Mean (and SD), %</td>
</tr>
<tr>
<td>Isolab no. 1</td>
<td>8.1(0.96)</td>
<td>11.9</td>
<td>15.8(6.34)</td>
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<td>Isolab no. 2</td>
<td>6.3(0.37)</td>
<td>5.9</td>
<td>7.0(0.63)</td>
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<tr>
<td>Helena no. 1</td>
<td>8.6(0.36)</td>
<td>4.2</td>
<td>12.5(0.92)</td>
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<tr>
<td>Helena no. 2</td>
<td>10.3(0.95)</td>
<td>9.2</td>
<td>11.4(1.87)</td>
</tr>
<tr>
<td>CliniTech</td>
<td>20.6(0.80)</td>
<td>3.9</td>
<td>18.9(3.73)</td>
</tr>
<tr>
<td>Hemolysate no. 1</td>
<td>8.2(0.70)</td>
<td>8.5</td>
<td>8.2(1.25)</td>
</tr>
<tr>
<td>Hemolysate no. 2</td>
<td>10.4(0.22)</td>
<td>2.1</td>
<td>10.9(1.73)</td>
</tr>
</tbody>
</table>

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Fig. 1. Linearity of the three HbA1c methods

Samples containing known fractions of "fast" hemoglobin were prepared (see Materials and Methods) and analyzed in duplicate or triplicate by each method. Each point represents the mean of duplicate or triplicate observations. The least-squares linear regression of measured fraction against expected fraction is represented for each procedure.
it. Because HbA1 and HbA1c values are clinically equivalent (1), the Isolab values are stable for at least 48 h at 4 °C, and clinically useful values have been reported by the Isolab procedure by others (4–8), we believe the Isolab procedure will be of value in monitoring long-term glycemic control in diabetic patients.

Several factors not addressed in this study can cause inaccurate values for HbA1c or HbA1 to be obtained by cation-exchange chromatography. Variations in the column temperature can cause a relative change of up to 5% per degree centigrade (9–14). The temperature in our laboratory ranged only from 21.5 °C to 24.5 °C over the period of this study. Because all assays were done at the same time, we doubt that temperature fluctuations affected our comparison data. The presence of HbF can falsely increase HbA1 (10), and the presence of HbS or HbC may produce falsely low values when the slow-eluting hemoglobin fraction is used in the denominator to calculate percent HbA1 (15, 16). Patients with chronic renal failure have falsely increased HbA1 values because of co-elution of carbamylated hemoglobin with HbA1 (17).

All the available methods for measuring HbA1 have difficulties. There is no standard available nor is there a quality-control material of defined accuracy. It is also not clear what molecular species are being measured by the various techniques (18). Nevertheless, clinically useful data can be obtained with this test. Although further development of methods and standards should continue, our data indicate that analysis of HbA1 by the Isolab procedure is practical for the routine clinical laboratory and should aid in the management of the diabetic patient.

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References
Liquid-Chromatographic Measurement of Cyanocobalamin in Plasma, a Potential Tool for Estimating Glomerular Filtration Rate

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We describe a method for measuring unlabeled cyanocobalamin in human plasma, based on its absorbance at 368 nm after reversed-phase sample-cleanup on a cartridge filled with octadecylsilylated silica followed by "high-pressure" liquid chromatography. The diminution of the cyanocobalamin concentration in plasma follows first-order kinetics after about 30 min and may be used to calculate the glomerular filtration rate.

The classical method for measuring the glomerular filtration rate (GFR) requires a constant intravenous infusion of inulin and accurate urine collection and plasma sampling. The disadvantages of the inulin clearance method are well known: the considerable burden on the patients, the possibility of bacterial infection during the bladder catheterization, the lengthy time required, and the tedious chemical analysis for inulin (1). With the aim of simplifying the evaluation of the GFR, workers have applied other substances, such as ⁵¹Cr-EDTA (2–5) or ¹⁵⁶Co/⁶⁰Co-cyanocobalamin (6, 7). These made it possible to calculate, after one injection ("single shot"), the clearance of the test substance from measurements of three or four subsequent concentrations in the blood, the rate of diminution being used to calculate the clearance ("slope technique"). Use of radioactive materials such as are required in the slope technique is undesirable.

We report here a procedure for quantifying cyanocobalamin in plasma by "high-pressure" liquid chromatography (HPLC). The results for the clearance of cyanocobalamin indicate this substance to be a tool for the evaluation of the GFR.

Materials and Methods

Apparatus

The HPLC apparatus was that of Knauer (Berlin, F.R.G.): a Model 52.00 pump, a Model RH 17 (100-µL loop) injection system, a 250 × 4.6 mm column filled with 5-µm Lichrosorb SI 60 (E. Merck, Darmstadt, F.R.G.), and a Model 85.00 variable-wavelength detector. Column temperature (30 °C) was controlled with a Model 60.00 oven (Knauer). Sep-Pak C₁₈ cartridges (Waters Associates, Milford, MA 01757) were applied for the reversed-phase cleanup.

Reagents and Standards

Methanol, bromcresol green, sodium cyanide, trichloroacetic acid (TCA), and cyanocobalamin (p.i. grade Cytobion, 2000 and 5000 µg) were of analytical grade, except as noted, from E. Merck, Darmstadt, F.R.G. Cyanocobalamin as a standard for the analytical work was supplied by Sigma, München, F.R.G.

Solutions of sodium cyanide (1 g/L), TCA (3 mol/L), bromcresol green (20 mg/L) and 10, 20, 30, and 50 mg/L cyanocobalamin and dicyanocobalamin (-----)