

An Automated "High-Pressure" Liquid-Chromatographic Assay for Hemoglobin A_{1c}

Graham Ellis,¹ Eleftherios P. Diamandis,¹ Esther E. Giesbrecht,¹ Denis Daneman,² and Lynn C. Allen³

An automated "high-pressure" liquid-chromatographic assay for hemoglobin A_{1c} is described. We use a 45-min incubation in acetate buffer (pH 5.5) to eliminate labile glycosylated hemoglobins. In this automated system conventional modules are used but it incorporates a solvent-switching valve to select either of two buffers, which differ in pH and NaCl concentration. The chromatographic column contains "polyCAT" (a weak cation-exchanger, polyaspartic acid linked to silica). Run time is 14 min per sample. The method is precise and results correlate well with those by other ion-exchange procedures.

Additional Keyphrases: *cation-exchange column chromatography · glycosylated hemoglobins · abnormal hemoglobins · reference interval · diabetes*

It has become apparent in recent years that proteins may become modified with carbohydrate moieties post-translationally, by enzymatic or non-enzymatic processes. One of the most reactive physiologic substances in the non-enzymatic process is glucose, which binds covalently to amino groups (1). Glucose adducts of hemoglobin (Hb) are properly termed glycosylated hemoglobin (GHb) (2).⁴ Most of the GHb is modified at the N-terminal valine residues of the β -chains (3). The initial reaction of glucose with this residue produces an aldimine or Schiff base, often referred to as "labile" GHb because the reaction is slowly reversible at physiologic pH (4, 5). The aldimine undergoes slow rearrangement to produce a stable GHb in which a 1-deoxyfructose moiety is linked to the Hb via a ketoamine linkage. Other GHb is modified at alternative sites such as lysine residues on both α and β chains (3) but these sites are less reactive, presumably because of the pK of the amino groups or because of steric factors. There is clinical interest in GHb because its measurement in diabetes mellitus affords a useful index to mean or "integrated" blood glucose concentration over the days or months that the erythrocytes have been in circulation (6-8). Methods for GHb measurement have been expertly reviewed (4, 9). They all rely on one or the other of two principal differences between Hb and GHb: differences in charge, exploited by ion-exchange chromatography, electrophoresis, or isoelectric focusing; or the presence or absence of the sugar moiety as detected chemically after hydrolysis or by affinity chromatography directed against the 1,2-cis-diol groups of the sugar moiety.

On electrophoresis the GHb moves with the "fast" Hb fraction (HbA₁). Ion-exchange chromatography gives better

resolution than electrophoresis, and the A₁ fraction is resolved into four or more components (9-12), comprising mainly HbA_{1a1} (Hb-fructose 1,6-diphosphate adduct), HbA_{1a2} (Hb-glucose 6-phosphate adduct), HbA_{1b} (probably a deamidation product of Hb), and HbA_{1c} (primarily GHb modified at the β -terminal valines). However, not all the material that is eluted in the HbA_{1c} peak binds to affinity columns, indicating that the peak does not represent a single component (3). Moreover, affinity columns react with material making up about 5% of the HbA₀ peak obtained after purification by ion-exchange, which probably reflects Hb that is glycosylated at sites other than β -chain N-terminal valines (3). Despite these differences in the fractions detected by the different methods, there is a useful clinical correlation between GHb results obtained by ion-exchange and by affinity column or colorimetric methods, unless molecular charge is affected by N-terminal carbamylation in uremia, acetylation in chronic therapy with salicylate, penicilloylation in therapy with antibiotics, the presence of 5-deoxyxylulose 1-phosphate adducts in alcoholics, or amino acid modifications in some hemoglobinopathies, as reviewed by Gabbay (13) and others (4, 9).

Several authors have described liquid-chromatographic (HPLC) systems for separating hemoglobins. All have used cation-exchange chromatography on various supports such as BioRex-70, which contains a weak carboxylic acid function (14-18); polyCAT, which also has a carboxylic acid function in the form of polyaspartic acid linked to silica (19, 20); a silica-based carboxymethyl polyamide solid phase (21-24); or "Micropearl," an ion-exchange resin of undisclosed structure, used with the Daiichi analyzer (25, 26). Columns of BioRex give less-satisfactory resolution than do silica-based columns. Flow rate is limited by the compressibility of the resin and its non-uniform size. It may be necessary to reverse the columns frequently because expansion and contraction of the resin in response to the changing ionic composition of the mobile phase causes distortion of the column bed and deterioration of chromatographic performance. BioRex-70 columns for HPLC are usually prepared in the laboratories in which they are used because they currently are not available commercially; this limits their utility for routine laboratories. In view of the excellent precision reported in the literature for HPLC methods for HbA_{1c}, and the improved resolution of the newer column-packing materials such as polyCAT as compared with that of BioRex-70, we chose to adapt the method of Ou et al. (19), which was designed for the separation of variant hemoglobins to the routine analysis for HbA_{1c}. Our method has twice the throughput of that of Ou et al. and does not require an HPLC gradient-generating accessory. We describe here our method and illustrate the critical variables affecting separation of HbA_{1c} by HPLC.

Materials and Methods

Equipment

We use a liquid-chromatographic system with spectrophotometric detection at 405 nm. This comprises a wisp™

¹ Department of Biochemistry and ² Division of Endocrinology, Department of Pediatrics, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.

³ Department of Clinical Biochemistry, Toronto General Hospital, Toronto, Ontario M5G 1L7, Canada.

⁴ Nonstandard abbreviations: Hb, hemoglobin; GHb, glycosylated hemoglobin; HPLC, "high-pressure" liquid chromatography.

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sampler, a Model 590 pump with Autochrom Model 101 solvent-select valve, and a Model 481 spectrophotometer from Waters Scientific Ltd., Mississauga, Ont., Canada, and a Model LCI-100 integrator from Perkin-Elmer (Canada) Ltd., Downsview, Ont., Canada. We use a 20-cm column of PolyCAT A with 5- μm packing and a 2-cm guard column from Custom LC, Inc., Houston, TX 77272-0351, and an in-line precolumn filter (Waters Scientific).

Reagents

Buffer A: 40 mmol/L phosphate, pH 6.30, containing 60 mmol/L NaCl, 5 mmol/L NaCN, and 0.5 mL/L Triton X-100. Dissolve 10.88 g of KH_2PO_4 , 7.02 g of NaCl, and 0.490 g of NaCN in approximately 1900 mL of water, adjust the pH to 6.30 with 5 mol/L and 0.5 mol/L KOH, and adjust the volume to 2 L. Recheck the pH. Filter through a Millipore Type HA 0.45- μm filter. Transfer to a 2-L polyethylene bottle containing a magnetic stir bar. While mixing continuously, slowly add 1 mL of Triton X-100 (alkylaryl polyether alcohol) from a pipette, with the tip placed just beneath the surface of the buffer. Leave the solution mixing for a further 5 to 10 min to dissolve all the Triton. De-gas the buffer by sonication in a waterbath for 10 min. This buffer is stable at room temperature for one week. Recheck the pH before use.

Caution. NaCN is toxic. Follow safety regulations concerning the safe storage, use, and disposal of buffers and HPLC effluent containing cyanide.

Buffer B: 40 mmol/L phosphate, pH 7.10, containing 200 mmol/L NaCl, 5 mmol/L NaCN, and 0.5 mL/L Triton X-100. Dissolve 10.88 g of KH_2PO_4 , 23.4 g of NaCl, and 0.490 g of NaCN in approximately 1900 mL of water, adjust the pH to 7.10 with 5 mol/L and 0.5 mol/L KOH and dilute to 2 L. Filter, add 1 mL of Triton X-100, mix, and sonicate (as for Buffer A). This buffer is stable for one week at room temperature.

NaCl solution, 150 mmol/L. Dissolve 8.8 g of NaCl in 1 L of water.

Acetate solution for removal of the labile fraction of glycated hemoglobins. Dissolve 3.4 g of sodium acetate trihydrate and 3.3 g of NaCl in approximately 400 mL of water. Adjust the pH to 5.5 with 2 mol/L acetic acid and the volume to 500 mL. (Solution contains 50 mmol of sodium acetate and 113 mmol of NaCl per liter.) This solution is stable for two weeks at 4 °C.

Blood samples. Collect blood by venipuncture, using EDTA as anticoagulant. Blood may be stored at 4 °C for four days (27) to one week (9) before analysis.

Quality-control bloods. We found that commercially available lyophilized controls were unsuitable for quality-control purposes. The HPLC pattern was quite different from that for fresh blood, and samples often showed multiple abnormal tailing peaks. Consequently we prepared our own controls as follows:

Pipette into 12 \times 75 mm polystyrene tubes 200 50- μL aliquots of blood from each of three individuals with HbA_{1c} approximately 5%, 10%, and 15% of total hemoglobin, add 2 mL of 150 mmol/L NaCl to each, mix, and incubate for 5 h at 37 °C. Centrifuge at 1400 $\times g$ for 10 min, decant the saline, and stopper the tubes. Transfer the washed erythrocytes to a -70 °C freezer. On the day the samples are to be used, add 4 mL of Buffer A, mix, transfer to a 5-mL syringe, and filter through a disposable Millex®-HV 0.45- μm filter unit (Millipore Ltd., Mississauga, Ont., Canada).

Samples are stable for at least six months at -70 °C. Storage at -10 to -20 °C is unsatisfactory (9). We have not evaluated an alternative method of stabilizing the Hb by

use of ethylene glycol (28), but laboratories that do not have access to -70 °C freezers may choose to assess this method.

Procedure

Mix the blood sample and pipette 50 μL into a 12 \times 75 mm polystyrene tube. Add 2 mL of acetate solution, mix, and incubate for 45 min in a 37 °C air oven to remove the labile fraction of glycated hemoglobin. Centrifuge the suspensions at 1400 $\times g$ for 10 min. Decant the acetate. Add 4 mL of Buffer A. Mix well to resuspend the erythrocytes, which are rapidly lysed by the Triton X-100 in the buffer. Transfer the hemolysate to a 5-mL syringe, filter through the disposable filter unit, and collect about 1.5–2.0 mL in a wisp sample vial and the remainder in a 12 \times 75 mm polystyrene tube as a back-up hemolysate. Cap the wisp vial and load it on the sampler. Cap and store the back-up hemolysate at 4 °C for as long as four weeks, if necessary.

Chromatographic Conditions

(For conditioning of a new column see later.)

Prime the chromatograph and solvent select unit with Buffers A and B. Equilibrate the column with Buffer A at a flow rate of 1.3 mL/min for 15–30 min. Program the 590 pump to send 0.1-min pulses to the solvent select unit to switch to Buffer B at 0.5 min after injection, and return to Buffer A at 6 min, both at a flow rate of 1.3 mL/min. Use a short piece of tubing between the solvent select valve and pump, to minimize the dead volume between the two units. Program the run times of both wisp sampler and integrator to 14 min. Program the 590 pump to slow its flow rate to 0.1 mL/min at 30 min after the injection. This command will conserve buffer and will take effect only at the end of the run because the timer is normally reset after each injection. Set the spectrophotometer to 405 nm. Select an area-percent plot on the integrator with optimum integration parameters. We find that with a 1-V input on the LCI-100, the optimum parameters are: peak width value 6, area sensitivity 30, baseline sensitivity 8, baseline drawn through base points, print tolerance >3% of total area, chart speed 5 mm/min, attenuation 32 giving full-scale deflection of 0.032 A, offset 5%, force baseline at 13.9 min. Select tick marks on the printout to denote the areas included in the calculated area, because these are a valuable form of quality control—see *Results* section. Program the wisp sampler to inject 20 μL of each sample. Load the wisp carousel with vials containing the hemolysates. Run high, medium, and low quality-control samples at the start and end of each run. The chromatographic patterns should resemble those shown in Figure 1.

Preparation of a New Column

When a new column is first used, wash it with distilled water for 1 h, then with Buffer A for 1 h. Test a quality-control sample with 14–16% HbA_{1c} six to 10 times, using the recommended procedure, until the chromatographic pattern shows good resolution of the HbA_{1c} peak (Figure 1) and acceptable accuracy and precision are achieved. If this does not occur, make minor adjustments to the composition of Buffer A or adjust the column temperature as discussed later.

Results

Development of Method

Choice of column. Two columns were tested initially. One was a 6 \times 125 mm column packed with a methacrylic acid copolymer (10- μm particle size) having weak (unspecified) cation-exchange properties, manufactured by Sekisui

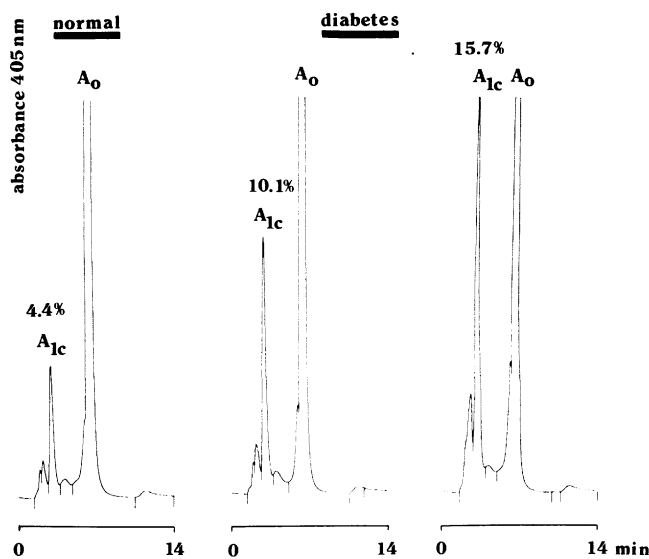


Fig. 1. Chromatographic separations of HbA_{1c} in blood samples from one normal and two diabetic subjects

Chemical Co. and supplied by Waters (Canada) Ltd. The second was the column of polyCAT from Custom LC Inc. The chemical structure of polyCAT is discussed in ref. 20. Columns were run at a flow rate of 1.3 mL/min with pressures of 6.21 to 8.28 MPa (900–1200 lb./in.²) as recommended by the manufacturers. Early experiments gave similar resolution on the two columns with slightly different chromatographic conditions. However, the polyCAT column cost approximately half as much and consequently we used this column in most of our development work. After the method had been in routine use for several months, we experienced some difficulties with a batch of polyCAT columns, which prompted us to test a Protein Pak SP 5PW 7.5 × 75 mm sulfopropyl column from Waters Scientific. Different buffer systems were required for this column and the conditions and results will be briefly presented later.

Column life. After 200–600 injections, resolution deteriorates; the A_{1c} peak becomes broader and tails. When this occurs, the integrator does not resolve the HbA_{1c} peak from that which immediately follows it, and this peak is included in the A_{1c} area (as indicated by the positioning of the "tick" marks on the integrator). This results in poor reproducibility and generally an increase in the fraction of the Hb reported as the HbA_{1c}. The error may represent 0.5–3% of total hemoglobin.

Choice of buffers and time sequence of buffer changes.

Note: It has been our experience with columns supplied by Custom LC Inc. that different columns prepared from the same batch of packing material have very similar properties. However, those prepared from different batches of polyCAT may have sufficiently different retentivity to necessitate minor changes in the pH or NaCl concentration of Buffer A or necessitate the use of increased column temperature to produce satisfactory resolution of HbA_{1c}.

Fast hemoglobins (HbA_{1a}, HbA_{1b}, HbA_{1c}) are less readily retained by cation-exchange resins than is HbA₀. We did not have a gradient apparatus on our HPLC, so we wished to use conditions under which the fast hemoglobins were resolved with use of a suitable buffer (Buffer A), after which the remaining hemoglobins (usually mainly the A₀ fraction) were rapidly eluted by use of a high salt/pH buffer (Buffer B). Time is then needed to re-equilibrate the column in preparation for the next injection. Using the recommended buffers, and with the equipment we describe, the selection of

Buffer B at 0.5 min gives the chromatography shown in Figure 1 (some time is needed to fill the pump and column with the new buffer). Selection of Buffer B after 0.5 min delays the elution of the hemoglobin A₀ peak, but does not improve resolution. Approximately 5–6 min of elution with Buffer B at the recommended flow rate of 1.3 mL/min is required to elute the A₀ peak. An 8-min wash with Buffer A re-equilibrates the column in preparation for the next sample. If this time is shortened, re-equilibration does not take place and the fast Hb fractions elute too early. By use of relatively high pH and high-molarity Buffer B and with buffer changes at 0.5 and 6.0 min, the composition of Buffer A can be critically adjusted. At 60 mmol/L NaCl, pH has a marked influence on elution (Figure 2). At pH 6.1 and 6.2 the retention times of HbA_{1a}, HbA_{1b}, HbA_{1c} are increased, resolution of HbA_{1a} and HbA_{1b} is improved, but the HbA_{1c} has not completely eluted before HbA₀ elutes. [The small unknown peak that follows the HbA_{1c} is not seen. It probably co-elutes with HbA₀ under those conditions, because a similar HbA_{1c} value was obtained at pH 6.2 as at pH 6.3.] In contrast, if the pH is increased to 6.4, the retention time of all hemoglobins is shortened, leading to poor resolution. The NaCl content of Buffer A is also critical. Even at a pH of 6.4, Buffer A containing only 20 mmol/L NaCl does not cause complete elution of the HbA_{1c} before the A₀ is eluted. During early experiments we tried buffers containing no cyanide, but the chromatography was much less

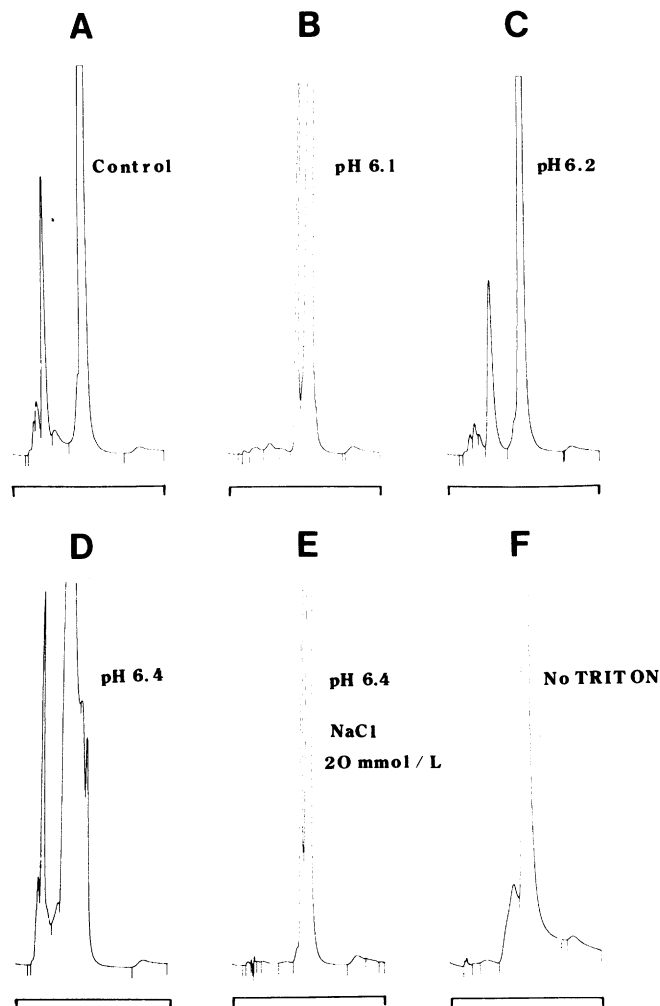


Fig. 2. Effects of pH, NaCl concentration, and Triton detergent on the chromatographic separation of HbA_{1c}

(A) Recommended method. (B–D) As A, but pH of Buffer A varied as shown. (E) As A, but pH and NaCl concentration of Buffer A altered. (F) As A, but Triton omitted from Buffers A and B. In each case, bar indicates 14-min run time

satisfactory. The A_0 peak was much sharper and showed less tailing with use of cyanide. Triton X-100 was included to prevent protein and lipid buildup within the system (29). At concentrations of 0.5 mL/L, but not 0.1 mL/L or less, it improved resolution and peak sharpness (Figure 2F). At 2 mL/L and greater, the increased concentration caused foaming.

Removal of the labile fraction of glycosylated Hb. In our early experiments, we added 2 mL of 150 mmol/L NaCl to 50 μ L of blood, mixed, and incubated at 37 °C for 5 h to remove the labile fraction. Bannon (30) showed that 200 mmol/L acetate, pH 5.5, removes the labile fraction in 30 min at 37 °C. We wished to use a buffer such that the residual buffer on the erythrocytes after decantation would negligibly affect the pH of our liquid-chromatography system. If the concentration of the pH 5.5 acetate is lowered to 10 to 100 mmol/L, there is some hemolysis. We therefore diluted the 200 mmol/L acetate with three volumes of 150 mmol/L NaCl to maintain a suitable osmolality. When we obtained a sample from a patient with poorly controlled diabetes mellitus and incubated it in the acetate buffer containing NaCl that we recommend, we observed a decrease in measured HbA_{1c} from 19.3% (without incubation) to 16.2, 16.0, 15.9, 16.0, 16.0, 16.2, and 16.0% after 15, 30, 45, 60, 120, 180, and 240 min at 37 °C in an air oven. Blood samples from the same patient gave values of 16.1% after 5 h incubation at 37 °C with 150 mmol/L NaCl. Paired blood samples from 10 diabetic patients were treated with either saline at 37 °C for 5 h or acetate for 45 min in a 37 °C air oven. Mean values for HbA_{1c} were 10.87% and 10.90%, respectively.

Hemolysate preparation. The method described is fast and convenient. We chose to wash the serum proteins from the erythrocytes to minimize the protein load on the column and eliminate any possible interference by bilirubin (9). We chose a simple wash with 40 times the blood volume rather than the traditional two washes with twice the blood volume, to shorten the labor time. We chose to hemolyze with Buffer A (containing Triton X-100) so that the pH of the hemolysate would resemble the chromatographic buffer into which it was injected. We wished to avoid a separate extraction with organic solvent (8, 14, 15) to remove the erythrocyte membranes after hemolysis. Filtration through Millex filters is rapid and effective. One Millex filter (22 mm in diameter) will filter the erythrocyte membranes from 50 μ L of lysed blood with a normal hematocrit before the filter pores become plugged.

Effect of sample volume. Variations in the amount of hemoglobin injected into the system have little effect on the result. If the patient is grossly anemic, or the blood was not adequately anticoagulated and a very dilute sample of erythrocytes is sampled from around a clot, then an inaccurate result may be obtained. Such hemolysates can often be identified visually. The total area calculated by the integrator may also be examined, and should be within $\pm 30\%$ of the mean area. Prepare more concentrated hemolysates from such patients by use of more erythrocytes or less buffer. At very low hemoglobin concentration, the integrator distinguishes peak boundaries less readily. If the hemoglobin is greatly increased, the column is overloaded with protein and the quality of the chromatographic separation deteriorates. When the volume of a hemolysate from a normal subject was varied between 5 and 100 μ L, optimum separation was achieved with 10- to 20- μ L injections.

Effect of temperature. We routinely ran our system at room temperature (24–28 °C) without temperature control of the column. We kept the column away from exhaust vents in equipment and observed no detectable change in quality-control values with temperature in routine use over a six-

month period. To investigate the effect further, we placed a column in a water bath and varied the temperature between 15 and 30 °C. At higher temperatures, retention times decreased for both HbA_{1c} and HbA₀ and there was a slight increase in the percentage of measured HbA_{1c} (Figure 3). However, we have recently found it necessary to run some later columns, prepared from a different lot number of packing material, at 30 °C to obtain satisfactory resolution and quality-control values.

Precision. Quality-control samples prepared as described above were analyzed 20 times to obtain within-batch precision and subsequently over the course of 24 batches during 23 weeks. Results are shown in Table 1.

Stability of hemolysates. Hemolysates were analyzed and subsequently stored at room temperature (approximately 25 °C) in the WISP sampler for 24–30 h before re-analysis. Results of the second analysis were not substantially different from those obtained initially. Longer storage at room temperature caused the hemolysates to turn brown, with deterioration in the quality of the chromatography. In contrast, the prepared hemolysate was remarkably stable at 0–4 °C. Over the course of several weeks, we re-assayed the "back-up" aliquots of groups of filtered hemolysates (see *Methods*) that had been stored at 0–4 °C after their preparation. Mean values on the day of preparation or several weeks later are shown in Table 2. Differences between individual analyses were usually small (<0.3%). The HbA_{1c} fraction in the hemolysate appeared to increase slightly but not significantly on storage.

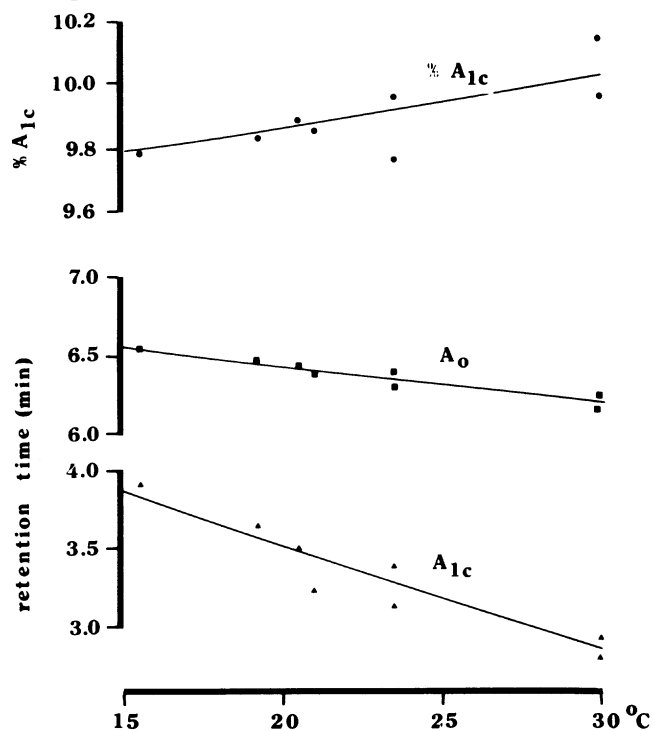


Fig. 3. Effect of temperature on retention time and quantification of HbA_{1c}.

Table 1. Precision of the Assay

	No. assays	Mean	SD	CV, %
<i>Within-batch</i>				
Patient A	20	4.32	0.10	2.3
B	20	10.03	0.11	1.1
C	20	15.60	0.18	1.1
<i>Between-batch</i>				
Patient A	24	4.18	0.12	2.9
B	24	9.88	0.13	1.3
C	24	15.80	0.05	0.3

Table 2. Stability of Hemolysates at 0–4 °C

Time between first and second analysis (weeks)	1	2	3	5	6
No. of samples in batch	46	19	8	27	12
Mean HbA _{1c} per batch, %					
Fresh	9.58	9.24	8.76	8.92	9.83
Stored	9.66	9.33	8.77	9.00	9.95

Reference values. HbA_{1c} was measured in 42 healthy laboratory staff members (20 men, 22 women), ages 18 to 65 years (mean 33.9); The mean HbA_{1c} was 4.82% (SD 0.42%); the mean values for men and women were not significantly different. Plots of cumulative frequency (probability scale) vs HbA_{1c} value were linear, indicating gaussian distribution of the values. The 2.5th and 97.5 centiles from this plot were 3.9% and 5.7%. The mean ±2 SD limits were 4.0% and 5.7%. Suitable reference values are 3.9% to 5.7%.

Comparisons with other methods. We analyzed 136 samples by the present procedure and for HbA_{1c} by a minicolumn procedure (Bio-Rad Labs., Richmond, CA 94804). Comparison was also made between the HbA_{1c} results for 170 samples analyzed by the present procedure and with two automated HbA_{1c} analyzers (Model HA-8110; Kyoto Daiichi Kagaku Co., Kyoto 601, Japan). In addition, we analyzed 70 samples by the present procedure and calculated the total fraction of HbA_{1a}, HbA_{1b}, and HbA_{1c} as "total HbA₁". These samples were tested by a minicolumn procedure for total HbA₁ (Fast Hb Test System; IsoLab Inc., Akron, OH 44821) modified to include a saline incubation to remove the labile fraction (31). The results are shown in Figure 4.

Abnormal hemoglobins. The gradient system of Ou et al. (19), from which this method was derived, was designed to resolve hemoglobin variants. Because our system was designed to measure glycosylated hemoglobins with maximum throughput, resolution of abnormal hemoglobins (Figure 5) was not as good as in their method. Hemoglobin F (Figure 5A) is not resolved from HbA_{1c} in this system, but very few patients over two years of age have more than 0.8% of this hemoglobin. Heterozygotes for HbS show a split "A₀" peak, and the HbA_{1c} is very much lower than that expected for the level of diabetic control as assessed by other criteria (Figure 5D). We have assayed blood from one patient with both diabetes and the rare benign hemoglobin D. In that patient we observed 7.4% HbA_{1c} and a second peak immediately following the HbA_{1c} (presumed to be glycosylated HbD), which

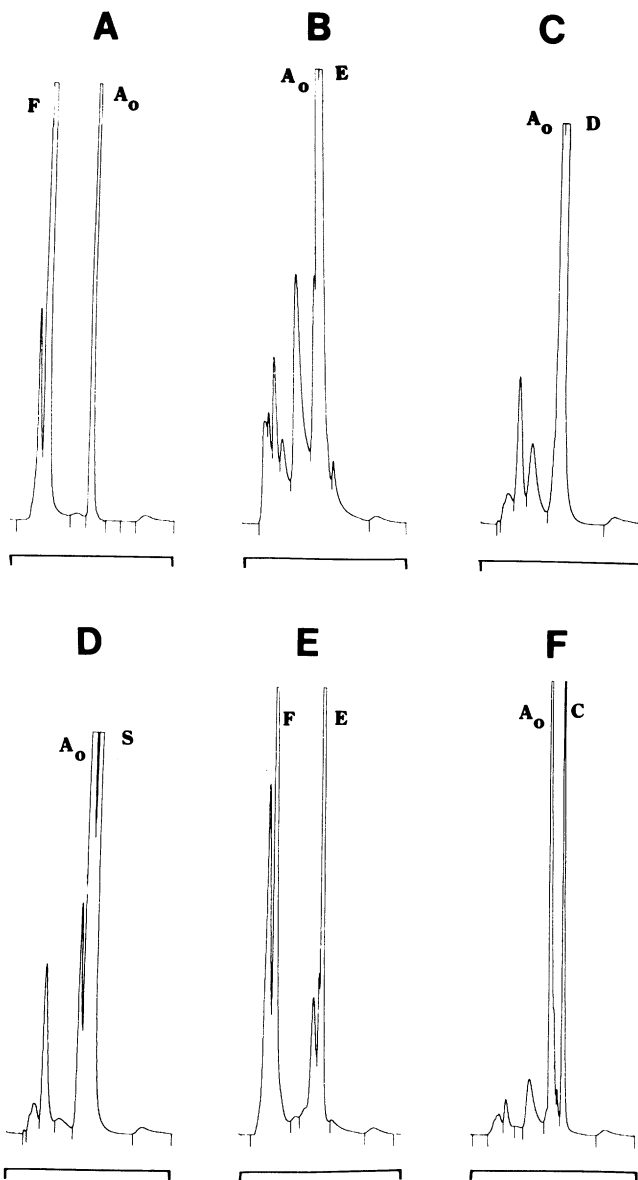


Fig. 5. Chromatographic separations of hemoglobins from patients with abnormal hemoglobins

(A) Neonate with predominance of HbF. (C) Patient heterozygous for presence of benign HbD. (D) Patient with sickle cell trait. (B, E, F) Patients with various other hemoglobinopathies as shown. In B and C, the integrator detected the HbE and HbD peaks as separate from the A₀ peak, although they appear fused at this level of attenuation. Bar indicates 14-min run time

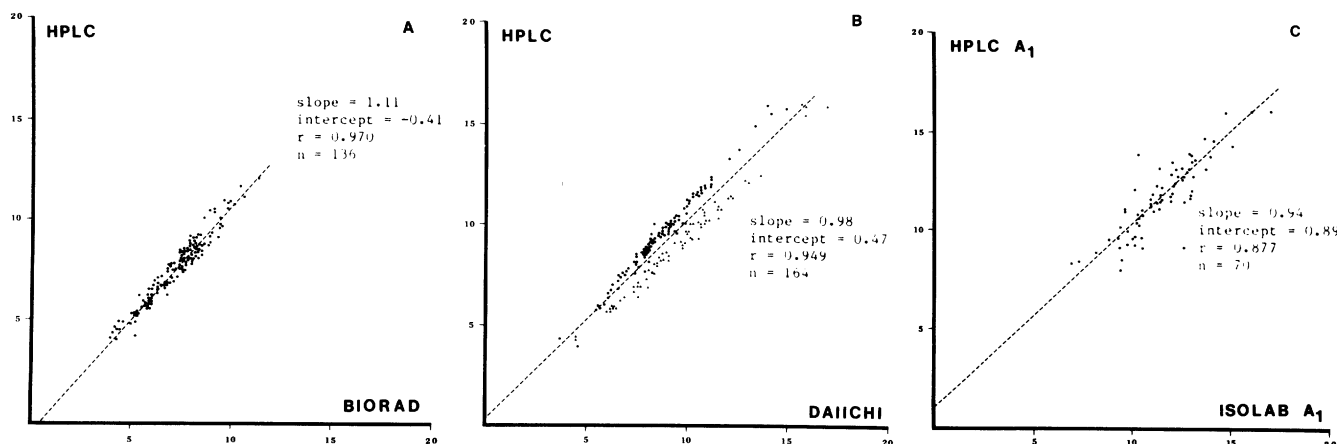


Fig. 4. Results by the present method compared with those of other procedures

HbA_{1c} as measured by the present method (HPLC) compared with (left) the method of Bio-Rad, (middle) the Daiichi A_{1c} analyzer (results obtained on two Daiichi Instruments), and (right) HbA₁ by HPLC compared with that by the Isolab procedure

composed 7.2% of the total Hb and which was increased to 8.8% on repeat analysis immediately after the sample of the patient's erythrocytes was incubated in 56 mmol/L glucose for 5 h at 37 °C. The remaining chromatograms in Figure 5 illustrate the resolution we achieved for samples of blood with abnormal hemoglobins.

Values in patients with thalassemia. We also had occasion to study 26 samples from patients with β -thalassemia, who were attending the outpatient clinic. None had diabetes. The chromatographic separation appeared normal, but the mean HbA_{1c} measured by the present method was 7.81% (SD 1.20). We do not know whether the increased HbA_{1c} was real or whether it was artefactually increased by the increased HbF in these patients; however, in view of this possibility, caution will be necessary in the interpretation of results for HbA_{1c} produced by this method in those rare patients who are afflicted with both thalassemia and diabetes mellitus.

Use of an alternative cation-exchange column. We have modified our method for use with a Waters SP 5PW column, which contains a rigid hydrophilic resin with sulfopropyl cation-exchange function. We found it necessary to use a flow rate of 1.0 mL/min and to reduce the pH of Buffer A to 6.00 and its NaCl concentration to 50 mmol/L. Resolution and precision were not as good as with the polyCAT column, and the HbA_{1c} results were more sensitive to column loading than those obtained with the polyCAT column (Figure 6). Results correlated well with those obtained with polyCAT: HbA_{1c} (SP-5PW) = 0.989 HbA_{1c} (polyCAT) + 0.047; $r = 0.987$; $n = 97$.

Discussion

This automated HPLC procedure for HbA_{1c} makes use of a recently developed column packing material in which polyaspartic acid covalently bonded to silica particles acts as cation exchanger. Resolution is better than with BioRex-70 (6, 14–17). The 14-min run time is similar to that of Toren et al. (21) and that of the Daichi HA-8110 analyzer, but is much shorter than others that have been described (8, 14, 22, 23), which range from 26 to 90 min.

The separation of HbA_{1c} from other Hb components with this system depends critically on buffer composition, particularly with respect to the pH and NaCl concentration of Buffer A. We have found that it may be necessary to make minor variations in pH (± 0.05 to ± 0.10), NaCl (± 10 mmol/L), or temperature (in the range 25–30 °C), to optimize chromatography for different lot numbers of column-packing material. From a review of the types of patterns seen under different elution conditions (Figure 2), we found it relatively easy to arrive at optimum separation conditions for a Waters SP 5PW column, which is based on a sulfopropyl ion-exchange resin rather than a polyaspartic acid-silica-based column, but this column had lower resolution and seemed more sensitive to hemolysate loading than the polyCAT column. From the data that we have presented, others may choose to adapt the method to alternative columns as these become available.

Like other HPLC systems (17, 21, 32, 33), the assay is precise, probably because of the high level of automation. Because the HbA_{1c} is calculated as a fraction of total hemoglobin, the absolute mass of Hb injected into the system is not critical and sample preparation can be simplified. Consequently, precision depends more on the quality of the chromatographic separation, the level of noise in the spectrophotometer, and the fine tuning of the integrator than on volumes pipetted.

Our reference values for HbA_{1c} are similar to those reported by others (3, 7, 14). Values in patients with

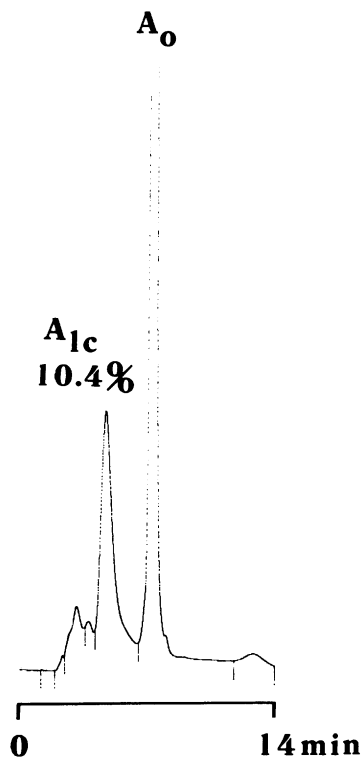


Fig. 6. Use of a Waters SP 5PW column in the analysis of blood from a diabetic patient

The sample was processed and run as in the regular method except that the flow rate was 1.0 mL/min and Buffer A was pH 6.00 with NaCl concentration 50 mmol/L

diabetes correlated well with those obtained by other ion-exchange procedures (Figure 4). Our experience supports the views of others (4, 6–8) that GHb provides information for the assessment of diabetic control that is unique and cannot be obtained from either clinical assessment or the examination of data on patients' plasma or urinary glucose.

Although this system is not designed to resolve abnormal hemoglobins, we have detected several during the past six months that the method has been in routine use, and the method seems useful in screening for abnormal hemoglobins in patients who may have HbA_{1c} values that are very different from those expected from the level of diabetic control as assessed by other criteria. Confirmation of the abnormality may be aided by operating the HPLC with Buffer A only over a 20–30 min run time. Late-eluting peaks are very broad, but HbA₀ is usually separated from other major components. Alternatively, laboratories with gradient systems could investigate these samples further by the method of Ou et al. (19). Hemoglobin variants differing from A₀ at sites that do not affect molecular charge cannot be resolved by HPLC ion-exchange methods, but reversed-phase separation of tryptic digests is proving to be a very useful procedure for their identification (34).

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