Sources of Variation in the Column-Chromatographic Determination of Glycohemoglobin (HbA₁)

Andries P. M. Schellekens,¹ Gerard T. B. Sanders,² Wayne Thorton,¹ and Theo van Groenestein¹

We studied the influence of many variables on the cation-exchange chromatographic determination of glycohemoglobin with minicolumns (HbA₁) and with macrocolumns (HbA₁α+β and HbA₁c). Minicolumns are most sensitive to buffer composition. The best conditions are pH 6.78 and sodium, potassium, and phosphate concentrations of 54, 8, and 36 mmol/L, respectively. In fact, there is no clear cut-off point for elution. A cyanide concentration of 1.5 mmol/L has no negative effects. For macrocolumns, the influence of temperature is demonstrated: 20 °C is optimal. A higher temperature caused results for HbA₁ to be higher. For minicolumns, the temperature effect corresponds to 1% HbA₁ per 1 °C, necessitating rigorous temperature control. The amount of sample applied also influences the results. Therefore a constant column load, chosen between 1.3 and 2.0 mg of Hb per milliliter of resin, is advised. Heparinized blood can be kept for three days at 4 °C; hemolyzed samples are best stored at −70 °C.

By determining glycosylated hemoglobinins (HbA₁) the degree of metabolic control in diabetes in the weeks preceding the blood sampling can be assessed (1-4). Different techniques for determination of HbA₁ have been described, such as cation-exchange chromatography (5-11), isoelectric focusing (12), colorimetric determination of the heoxietes linked to the hemoglobin (13, 14), and also a radioimmunoassay for one of the HbA₁ fractions (15). In the cation-exchange chromatographic procedures, a group of three glycosylated hemoglobin derivatives—HbA₁α, HbA₁β, and HbA₁c—collectively denoted HbA₁—are assayed as one fraction when small columns are used. For this purpose, “minicolumn” sets are marketed (16, 17). The largest of the glycosylated HbA₁ fractions, HbA₁c, can be separately determined on longer “macrocolumns” (1). The minicolumn procedures have some apparent advantages, such as simplicity, speed, and small sample volume. On performing this technique we noticed variations in our results that could be attributed to several external factors. Therefore we further investigated the effects of pH, ionic strength, and cyanide concentration of the elution buffer, and the working-temperature, column-load, and sample-storage conditions. Results by the minicolumn technique are compared with those by macrocolumn.

Materials and Methods

Resin

For all experiments involving home-made columns, Bio-Rex 70 (200-400 mesh), a weakly acidic carboxylic cation exchanger, was obtained from Bio-Rad Laboratories, Richmond, CA 94804 (catalog no. 142-5852).

The resin was precycled and equilibrated as follows: the dry resin was (a) floated and mixed in 0.5 mol/L NaOH for 30 min (1 g resin/10 mL), (b) washed with distilled water until the pH of the wash water was 7, (c) mixed with 0.5 mol/L HCl for 30 min, (d) washed again until the pH was 7, (e) mixed with 0.5 mol/L NaOH for 30 min, (f) washed once again to pH 7, (g) suspended in the desired elution buffer and allowed to settle, and the fines removed, and finally (h) washed with elution buffer in a 30 × 4 cm column until the concentration and pH of the emerging buffer were the same as that of the starting buffer. When processing 100 g of resin this way and eluting with a speed of 1 L/h, it may take up to a week before equilibration is reached, depending on the batch of resin used.

Columns

We filled two sizes of columns with the equilibrated resin. (a) Minicolumns, 6 × 0.7 cm, were obtained from Sarstedt B.V., Bladel 5531 AV, The Netherlands (article no. 102/2), fitted with a sintered-glass frit. Resin added to a height of 3.5 cm gave a bed volume of 1.4 mL. (b) Macrocolumns were 20 × 1 cm “Econo-columns” (Bio-Rad, cat. no. 737-2240), filled to a height of 15 cm, which gave a bed volume of 11.5 mL.

We also used pre-filled minicolumns (Quik-Sep Fast Hemoglobin Test System, Isolab, Inc., Akron, OH 44321; and columns from the Hemoglobin A₁ by Column Test Kit, Bio-Rad). These also contained the Bio-Rex 70 resin and had bed volumes of 1.7 and 1.3 mL, respectively.

Buffer

(a) For minicolumns. For the experiments on optimal conditions to be used for the minicolumns, we prepared a series of 12 different buffers containing phosphate concentrations of 31, 36, and 41 mmol/L, each at pH values of 6.70, 6.74, 6.78, and 6.82, and all at 22 °C. The primary sodium phosphate solutions (containing 4.28, 4.97, and 5.66 g of Na₂HPO₄·2H₂O per liter) were respectively mixed with the secondary sodium phosphate solutions (containing 5.52, 6.41, and 7.70 g of Na₂HPO₄·2H₂O per liter) to obtain the desired pH values. The buffer sodium concentrations, measured by flame photometry, varied for the four pH values of the phosphate concentrations of 31 mmol/L from 46.5 to 47.5 mmol/L, for the phosphate concentration of 36 mmol/L from 53 to 55 mmol/L, and for the phosphate concentration of 41 mmol/L from 59 to 61 mmol/L. The primary and secondary sodium phosphate solutions also contained, per liter, 1.5 mmol of KCN and 6.6 mmol of KCl. In the case of the pre-filled minicolumns, the buffers included in the kits were used as prescribed.

(b) For macrocolumns. The two elution buffers used were modifications of those used by Trivelli et al. (1).

Buffer I

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<th>Component</th>
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<td>KCl</td>
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Distilled water to 1000 mL

The pH of this buffer was 6.69 ± 0.005 at 20 °C. The total [Na⁺] was 55 mmol/L and [PO₄] was 40 mmol/L.
Buffer II

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<th></th>
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Distilled water to 1000 mL

The pH of this buffer was 6.42 (range 0.005) at 20 °C.

The total [Na⁺] was 196 mmol/L and [PO₄²⁻] was 150 mmol/L.

All pH measurements were made with a PHM 64 research pH meter (Radiometer A/S, DK 2400, Copenhagen, Denmark).

Sample Preparation

Blood was collected in EDTA- or heparin-coated tubes, in ice. The erythrocytes were washed three times with an equal volume of cold saline, then hemolysed with 0.5 volume of distilled water. The cell ghosts were extracted with 0.25 volume of CCl₄. After thorough mixing and centrifugation, the lysate (upper) layer was aspirated, divided into 400-μL portions, and stored at −70 °C in small polyethylene tubes. For storage experiments, temperatures of 4 and −20 °C were used.

Before use, the lysates were adjusted to 35 μg of Hb per microlitre with elution buffer. For both commercial kit procedures, samples were prepared as directed by the manufacturers (16, 17).

Chromatography

(a) Minicolumns. The minicolumns and elution buffers were equilibrated to room temperature (22 °C) for at least 1 h. Before use, 2 mL of the elution buffer was applied to the column and the pH of the effluent was checked. The columns were loaded with 50 μL of the 35 μg/μL lysate and 0.5-mL fractions were collected. The absorbances of the fractions were measured at 415 nm vs elution buffer, in a Zeiss PM6 spectrophotometer. Analytical recovery from the column was calculated from measurements of the absorbance of 50 μL of the lysate diluted to 20 mL with elution buffer.

(b) Macrocolumns. The temperature of the macrocolumns was controlled more rigorously. Ten columns were fitted in the bores of a specially constructed aluminium holder that contained a piping circuit connected to a thermostated waterbath. The whole length of the columns was visible through a Perspex plate, 5 mm thick, fitted on the front of the aluminium holder. The temperature of the holder remained constant and differed by less than 1 °C from that in the waterbath. The temperature inside the bores of the holder, registered during several runs by thermosensitive bi-metal sensors, showed a maximum variation of 0.5 °C. The columns were connected to a large buffer reservoir maintained at the same temperature as that of the holder. Thus, to test the effect of temperature on the chromatographic pattern, we could set and control different temperatures. Buffer I was run and the eluent controlled for pH. A volume of 500 μL of the lysate was then applied to the resin and allowed to drain into it. The elution buffer was pumped through at a constant flow rate of 0.42 mL/min per column. To minimize any day-to-day variations, one column was run for 30 min before the others, with a known hemolysate as reference sample, and 2-mL fractions were collected and spectrophotometrically measured as before at 415 nm. From the elution pattern of this column, fraction volumes for HbA₁₈+b and HbA₁₉ were established and used when eluting the remaining columns. At 20 °C the HbA₁₈+b fraction (1) was eluted with 24 mL of buffer and the slower HbA₁₉ fraction (2) with an extra 56 mL of the pH 6.69 buffer I. Once this second fraction had been collected, the reservoir buffer was replaced with the pH 6.42 buffer II. This eluted the remaining major Hb fraction (3) in 100 mL. On completion of the procedure, the reservoir was changed back to the pH 6.69 buffer I and the columns were re-equilibrated overnight with 200 mL of buffer I.

The absorbance of fractions 1, 2, and 3 was measured spectrophotometrically at 415 nm and the percentages of HbA₁₈+b and HbA₁₉ were calculated. Recovery from the column was checked as with the minicolumns.

A temperature of 20 °C was used in all routine measurements with the macrocolumn method.

Chromatography with Isolab and Bio-Rad minicolumns was performed according to the directions supplied by the manufacturers (16, 17).

Results

Analytical Variables

Elution patterns and column size. Elution profiles for macrocolumns and prefilled minicolumns from Bio-Rad and Isolab (Figure 1) showed that the macrocolumns separated the HbA₁ into two fractions, the first one the so-called HbA₁₈+b, the second one HbA₁₉ (6). After 80 mL the HbA₁₉ was quantitatively eluted. With the minicolumns, the glyco-hemoglobin fractions were eluted either in one peak or only partly separated, and at the prescribed elution volumes of 10 mL (Bio-Rad) and 4 mL (Isolab) elution was still incomplete (Figure 1). Therefore the results with the macrocolumn method were higher than those with minicolumns.

pH and ionic strength. The influence of pH and ionic strength of the elution buffer on the pattern of glycohemoglobins is illustrated in Figure 2. These experiments were performed with our home-made minicolumns. As could be expected, absorption of the hemoglobins by the resin diminished with increasing ionic strength and pH, when other variables such as temperature and amount of hemoglobin loaded onto the columns were kept constant.

Increasing the ionic strength by only 5 mmol of phosphate per liter and 7–8 mmol of sodium per liter, with no change in pH, resulted in an increase in the percentage of eluted HbA₁. When a buffer of pH 6.74 was used, the expected percentage HbA₁ (as determined with the macrocolumn method) could either be reached after 20 or 7.5 mL, depending on the ionic strength (Figure 2, Ib and IIIb). This effect was smaller at pH 6.70 with the same increase in ionic strength (Figure 2, Iia and IIIa).
On the other hand, there was a significant influence of pH variations at constant ionic strength, especially at the lower (31 mmol of phosphate per liter; 46–47.5 mmol of sodium per liter) and the higher (41 mmol of phosphate per liter; 60–62 mmol of sodium per liter) salt concentrations (Figure 2, I and III). In the case of the medium ionic strength, smaller differences between the cumulative percentages HbA1 were found, notably at pH 6.74 and 6.78 (Figure 2, II).

Cyanide concentration. The influence of the low cyanide concentration (1.5 mmol/L) in the elution buffer was studied on macrocolumns by running a normal and a diabetic hemolysate in five replicates in one series, using our buffer I or Trivelli’s developer 6 (I), both followed by our buffer II. The average percentages of HbA1 were 7.57 (SD 0.32) and 11.30 (SD 0.36) with our buffer I and 7.79 (SD 0.18) and 11.32 (SD 0.32) with Trivelli’s developer 6. These figures are not significantly different.

Temperature. (a) Minicolumns (Fast Hemoglobin Test System, Isolab Inc.). A pooled sample with a value of 10.9% HbA1, as established with the macrocolumn method at 20 °C, was chromatographed with the Isolab system and 0.5-mL fractions were collected. The columns were placed in a thermostated waterbath at temperatures of 11, 16, 20, 22, and 24 °C. Results are depicted in Figure 3. Only at 22 °C did the cumulative % HbA1, read at the 4-mL cut-off volume, correspond to the value with the macrocolumn method. Changes in temperature to 24 and 20 °C caused a 3% increase and a 2% decrease in % HbA1, respectively.

(b) Macrocolumns. Hemolysates from normal and diabetic persons were chromatographed at 16, 20, 24 and 28 °C with use of the aforementioned aluminum temperature-controlled block. Figure 4 shows an example of the resulting elution patterns for a hemolysate from a diabetic. The volumes necessary for elution of HbA1 and HbA1+HbA2 can be found there, where the increase in cumulative % HbA1 per milliliter of elution volume is minimal: the “plateau-values.” The corresponding % HbA1 values and the elution volumes are called “plateau-values” and “plateau-volumes,” respectively. From the curves it can be inferred that changes in the temperature at which chromatography is performed result in a number of effects:

- The higher the temperature, the smaller the elution volume at which the plateau-value is reached (plateau-volume).
- At higher temperatures, the nearly ideal plateau found at 16 and 20 °C gradually disappears and the plateau-slopes increase.

Fig. 2. Influence of buffer pH and ionic strength on chromatography of HbA1 at 22 °C on home-made minicolumns
Cumulative % HbA1 in a diabetic hemolysate vs elution volume as collected in 5-mL portions. Sample: 1.75 mg Hb/column. I: 31 mmol/L phosphate, 46.5–47.5 mmol/L sodium; II: 36 mmol/L phosphate, 53–55 mmol/L sodium; III: 41 mmol/L phosphate, 59–61 mmol/L sodium; a: pH 6.70; b: pH 6.74; c: pH 6.78; d: pH 6.82. The horizontal dotted line indicates the reference value (HbA1 = 9.8%) as determined by macrocolumn method
The effect was similar to that for the minicolumns, but smaller. On increasing the load by 1 mg/mL of resin, the maximum increase was 0.45% HbA1 and 0.6% HbA1 for pools A and N, respectively (Figure 5).

The ratio % HbA_{1+a+b+c} % HbA_{1+c} was calculated over the whole loading range. For pool A there was a steady increase from 0.40 to 0.50; for pool N it remained constant (0.55). The within-run CVs over the whole loading range were 1.6% (pool A) and 3.7% (pool N); at a constant load of 1.4 mg of Hb per milliliter of resin they were 1.2% (pool A) and 2.5% (pool N) (n = 10). This column load was used in all further experiments.

Between-run variability. (a) Minicolumns (Isolab). Between-run CVs for % HbA1 for samples stored at −70 °C and analyzed 12 times at 22 °C were 5.8% for a normal hemolysate with an average % HbA1 value of 7.97% and 6.3% for a hemolysate from a diabetic with an average % HbA1 value of 11.04.

(b) Macrocolumns. Between-run CVs for 16 single determinations at 20 °C during one month, for a normal sample stored at −70 °C, were 4.3% (HbA_{1+a+b+c}), 5.9% (HbA_{1+c}), and 5.2% (HbA_{1+a+b}); the corresponding average glycohemoglobin values were 7.38, 4.56, and 2.7, respectively. For a diabetic sample under the same conditions these CVs were 3.2% (HbA_{1+a+b+c}), 4.15% (HbA_{1+c}), and 4.6% (HbA_{1+a+b}); the corresponding average glycohemoglobin values were 11.3, 8.0, and 3.3, respectively.

Storage. Heparinized blood samples, taken from eight diabetic patients at day 0, were divided into two parts for storage, either as whole blood at 4 °C or for the preparation of hemolysates, which were stored at 4, −20, and −70 °C. All results were statistically compared to those at day 0 with Student's t-test for paired observations.

With use of minicolumns, the results for HbA1 analysis in whole-blood samples stored at 4 °C were unchanged after three days, but showed significantly lower values after seven days (p < 0.05). After 11 days of storage there had occurred a further decrease, averaging 1% HbA1. No significant change could be seen when hemolysed blood was stored at 4 °C for as long as 10 days or at −70 °C for 32 days. However, after 57 days at −70 °C results were significantly lower (p < 0.05).

For macrocolumn chromatography, whole heparinized blood could be stored for as long as seven days at 4 °C without significant effects; after 11 days a significantly lower result was found for the HbA_{1+c} fraction (p < 0.05), while the percentage HbA_{1+a+b+c} was unchanged.

Hemolysates stored at 4 °C and analyzed with macrocolumns showed a tendency for higher HbA1 values after four days, becoming significantly higher after 10 days (p < 0.05) with an average increase of 0.65% HbA1 (n = 8). This increase was completely the result of a larger contribution of HbA_{1+a+b+c}. Storage of hemolysates at −20 °C was also not useful: after two days an absolute increase of 0.8% and after 10 days of nearly 2% was found in the HbA1 result. At −70 °C, hemolysed samples could be stored for as long as 57 days with no significant change in the results of macrocolumn chromatography.

In Figure 6 we compare the fractionation patterns of a hemolyzed sample stored at −20 or −70 °C. In the sample at −20 °C a distinct increase was seen in the first fraction (up to 24 mL elution volume: HbA_{1+c}) and in a fraction eluted at 140–210 mL elution volume (HbA_{1+a+b+c}). The HbA1 fraction at 24–80 mL elution volume was almost the same in both samples, with values of 5.9% and 5.3% for the −20 and −70 °C samples, respectively.

### Values for Normal and Diabetic Individuals

With the macrocolumn method at 20 °C, normal mean values (n = 48) for hemolysates were for the % HbA_{1+a+b+c} 7.36 (SD 0.83), for the % HbA1c, 4.98 (SD 0.49), and for the % HbA_{1+a+b} 2.37 (SD 0.40). The results for % HbA_{1+a+b+c} and % HbA1c did not exceed 8.9 and 5.9, respectively.

In hemolysates of samples from diabetic patients (n = 22) the results of the % HbA_{1+a+b+c} % HbA1c, % HbA_{1+a+b} ranged from 7.6 to 17.8, 5.7 to 12.3, and 2.2 to 5.0, respectively. These results agree well with those in the literature (1, 4, 9).

There was a good correlation (r = 0.954) between the results.
obtained under optimized conditions with the macrocolumn and the Isolab minicolumn method. The linear regression equation was $y$ (Isolab minicolumn) = 0.9472$x$ (macrocolumn) + 0.03% HbA1.

**Discussion**

Negative experiences with minicolumn ion-exchange techniques for HbA1 determination, manifested as bad reproducibility, prompted us to start this investigation. Not only the columns prepared by us but also commercially available ready-to-use columns showed the drawback of inconsistent results. This inconsistency could be ascribed either to the specimen itself, in the way the sample is prepared and stored, or to the chromatographic method and the conditions at which it is performed—or to some combination of these. Therefore we studied in detail the variables which might influence the chromatography of HbA1 on Bio-Rex 70 columns.

Small variations in pH and ionic strength of the elution buffer can have great impact on the elution pattern as can be expected from the small differences in pl (12). Our results clearly quantify the related effects of both variables: the pH modulates the effect of a change in ionic strength and vice versa. Allen et al. (5) refer to these effects, as do Welch and Boucher (9), who, working with minicolumns of 3 mL bed volume, chose to use pH 6.74 and phosphate and sodium concentration of 40 and 62 mmol/L, respectively, a set of conditions comparable to that in Figure 2, IIIb. These conditions bear the disadvantage that at the reference value no plateau in the % HbA1 curve is found. For complete elution, reflected in a plateau in the cumulative % HbA1 value, the elution volume nearly always exceeds 15 mL. At a sample amount of 1.75 mg of Hb per column (1.3 mg of Hb per milliliter of resin), this would lead to insufficient absorbances in the HbA1 fraction ($A_{415} = 0.035$). So the choice of the buffer for this minicolumn procedure must be a compromise between small elution volumes and the extent to which a HbA1 plateau-value is attained. Our advice is to use the buffer with pH 6.78 and ionic strength of 36 mmol/L phosphate and 54 mmol/L sodium (Figure 2, IIc). Increasing the column load to 2 mg/mL of resin will then allow an elution volume of 10 mL.

Our results underline the fact that the use of minicolumns can only give acceptable reproducibility by careful preparation of the elution buffers and by adjusting the pH to within 0.005 pH unit.

Cyanide must be present in the buffer, to convert hemoglobin into the stable hemiglobincyanide, which not only is known to behave identically to oxyhemoglobin on cation-exchange chromatography but also shares with oxyhemoglobin an isoesthetic point at 415 nm. The main difference between Trivelli’s developer 6 (1) and our buffer I is the decrease in cyanide concentration from 6 to 1.5 mmol/L, according to Welch and Boucher (9). With either buffer, results for a normal and a diabetic sample are essentially the same.

Concerning temperature, our experiments clearly show (Figure 3) the enormous temperature effect when minicolumns are used, amounting to absolute differences in the final result of 1% HbA1 per 1 °C. Therefore, temperature control is important to improve the reproducibility of the minicolumn procedure and in our opinion temperature must be kept as nearly constant at 22 °C as possible.

In a number of publications this important aspect is not referred to (7, 9, 11, 20). Neither Trivelli et al. (1), Gabbay et al. (4), nor Pecoraro et al. (14) mention it, though they do correct for shifts in the elution pattern caused by temperature changes, by monitoring the absorbance of the eluate from one of their columns and adapting the elution volumes of the others in the series. Dix et al. (21), using minicolumns, noticed an increase in the % HbA1 of 1% per 3 °C. They propose simultaneous chromatography of a hemolysate with a known HbA1 content. This leads to an improvement, which is also seen in the results of Dods and Bolme (22), who report a between-run variability of 4%. According to Gabbay et al. (25) a constant working temperature of 28 °C leads to better results.

When we were preparing this manuscript a modified methodology for the Fast Hemoglobin Test System (Isolab) appeared (16), in which the manufacturer prescribed a narrow range for the working temperature: 22–24 °C.

For the macrocolumns, the effect of temperature was also demonstrated (Figure 4). A higher temperature leads to both faster elution to the HbA1 components and an increase in the plateau value. This suggests that absorbant capacity and resolution are both diminished, causing results for HbA1 to be higher.

Good plateaus in the elution profiles are obtained at 20 °C or lower and only then is a good separation found. Therefore, when one requires higher accuracy and precision, use of a macrocolumn method is preferable to a minicolumn, but only with the aforementioned temperature restrictions. The use of the thermostated metal block with Perspex front has proven to be ideal for hemoglobin chromatography, because not only temperature but also sample application and column performance can be controlled during the experiments.

The influence of variations in column load on the result for % HbA1 is less important than that of temperature. The effect is indeed related to column dimensions, and it was shown to be present both in minicolumns and macrocolumns, even when they were not overloaded. In general, an aliquot of diluted hemolysate is applied to the column, without correction for the Hb content. Consequently, the physiological Hb variation of 100–190 g/L will result in column loads ranging from 0.7 to 2.2 mg/mL of resin in the case of the minicolumns. This will lead to a maximal difference of 0.5% HbA1. The same variation of column load in the macrocolumn method gives a difference of 0.25% HbA1 (Figure 5).

We showed that standardization of the column load to 1.3 mg of Hb per milliliter of resin for the minicolumns and to 1.4 mg/mL for the macrocolumns decreases the within-run CVs to 2.5% or less. In general, a constant column load between 1.3 and 2 mg/mL will give acceptable results.

Good sample stability during storage is a necessity for the long-term quality control as well as for the reliability of results from stored samples. Reports in the literature concerning sample stability are not consistent, most probably because inter-series reproducibility can be maintained only when the
circumstances during which the experiments are performed are controlled accurately, as can be gathered from our observations concerning the effects of temperature, pH, and ionic strength. A stable hemoglobin sample, marketed as CO-Hb stored at 4 °C under carbon monoxide gas, is advocated for calibration purposes (21). However, it would be simpler to store a sample either in the form of whole blood or as untreated hemolysate, at 4 °C. Abraham et al. (11) and Pecoraro et al. (14) reported a good preservation of whole blood at 4 °C during a four-week period. Welch and Boucher (9), Dix et al. (21), and Bunn et al. (24) found it to be stable for at least seven days. Our results on storage of heparinized (whole) blood at 4 °C only confirmed these reports insofar as analysis was performed with macrocolumns.

An explanation for the significant decrease of HbA1 in whole blood at this temperature can be found in the results of Widness et al. (23). They noticed rapidly increasing HbA1c values parallel to acute changes in blood glucose; however, dialysis for 48 h yielded lower HbA1c values, probably owing to dissociation of weakly bound glucose from the glycohemoglobins. Possibly the same dissociation took place in our samples. A more speculative explanation for this effect can be found in the report of Schoos et al. (12). These authors demonstrate the existence of Hb forms (α2β2: asymmetric globin hybrids) with αβ values exceeding that of HbA1c (7.05 vs 6.95). When these forms are generated from glycoylated hemoglobins during storage, a decrease in the fast hemoglobin fractions will be the consequence.

The presence of HbA1c and HbA1c (Figure 6), normal circulating minor hemoglobins (6), contributes to the greater variability found with the minicolinum technique as compared with the macrocolumn method. In the latter they are completely excluded from the glycohemoglobin fractions. With minicolins they may partly elute with the HbA1c fraction and after storage of hemolysates at -20 °C their contribution increases, leading to more aberrant results.

In conclusion, we have shown that the ion-exchange chromatographic determination of glycohemoglobin is extremely sensitive to variations in pH, ionic strength, temperature, column load, and sample storage.

We prefer the use of the macrocolumn method, especially as a comparison method, because elution is more nearly complete, leading to more nearly accurate HbA1c results. Moreover, the separate measurement of HbA1c is more resistant to potential influences of storage. Finally the macrocolumn technique is more rugged, giving results with a better within-run and between-run variability.

We found a good correlation between % HbA1c values for macro- and minicolinum chromatography, so the latter can be used as a practical routine method if the variables are kept under good control. For self-made minicolins we would suggest a cation-exchange resin bed volume of 1.4 mL with a height of 3.5 cm; a sample load of 2 mg of Hb per milliliter of resin; an elution buffer of pH 6.78; sodium, potassium, and phosphate concentrations of 54, 8, and 36 mmol/L, respectively; and a working temperature of 22 °C. Even then, the macrocolumn technique will be indispensable as a reference method.

References