At present no method for glycohemoglobin (HbA1c) is automated on a main-line analyzer to allow joint measurement with other indicators of diabetic control such as glucose and cholesterol. We describe an adaptation of a latex-enhanced competitive immunoassay for quantifying HbA1c to the Dade International Dimension® analyzer. After a manual hemolysis step, HbA1c and total hemoglobin (Hb) are determined separately. The concentration of glycated β-subunit is obtained from the immunoassay, whereas Hb is assessed colorimetrically from a derivatized form. Both reactions were fully optimized for accuracy, precision, and specificity on the Dimension; stabilities of reagents and calibration were established; and potential interferences were assessed. The analyzer gave reliable results over the required clinical range of 1–15% HbA1c. Within-run and total assay variation were within 5% of the target CV limits, as determined by ANOVA with three representative sample pools across 20 days. Close agreement with an established HPLC procedure and a commercially available enzyme immunoassay was observed for 140 samples from clinically defined patient groups. Additional samples from patients with hemoglobinopathies (n = 20) demonstrated a more complex relationship between methods. We conclude that adaptation of the method for use with the Dimension analyzer is a valid method for quantifying HbA1c.

INDEXING TERMS: diabetes mellitus • hemoglobin variants

As is now recognized, intensive therapy to maintain good glycemic control can delay the onset and slow the progression of secondary complications in diabetes mellitus [1–3]. Furthermore, in most patients, measurement of blood glucose is not a sufficiently reliable indicator of glycemic control, the rapid fluctuations providing potentially misleading “snapshots” of events. Quantification of the proportion of glycation of hemoglobin, albumin, and more general serum proteins (fructosamine) has been proposed as an alternative marker to glucose [4–6]. Glycohemoglobin (HbA1c) is generally considered the most reliable indicator of glycemic control because it reflects the average concentration of blood glucose over the preceding ~90 days and consequently requires less frequent measurement to assess glycemic status [7, 8].

“Glycohemoglobin” refers to the carbohydrate–protein linkage on the N terminus of the β chains of hemoglobin, predominantly HbA in adults. HbA1c specifically refers to glucose addition and accounts for the majority of glycated species present [9]. HbA1c is usually expressed as a percentage of the total hemoglobin (THb) and can be measured by ion-exchange chromatography [10, 11], affinity chromatography [12], electrophoresis [13], or immunoassay [14–16]. These methods all demonstrate systematic differences between their reported values and thus do not give interconvertible results; rather, comparison of results determined by different methods requires both knowledge of the glycated species of hemoglobin measured and the use of appropriate calibrators and reference ranges. The presence of interfering substances will also produce different responses in these methods; e.g., the high proportion of circulating carbamylated hemoglobins in patients with chronic renal failure is responsible for positive interference in assessments of HbA1c by methods based on charge separation, whereas affinity chromatography and enzyme immunoassay are unaffected [17, 18]. The presence of genetically determined hemoglobin variants within a population, most commonly HbS, HbC, and HbE, has also been shown to influence the analysis of HbA1c by some types of HPLC and immunoassays [19].
Several HPLC instruments dedicated solely to the measurement of %HbA1c are available commercially [20], offering the user all the convenience of automated analysis. However, the immunoassay techniques, particularly the light-scattering examples, also offer advantages, in that they can be adapted equally to automated clinical chemistry analyzers [16], thereby avoiding the cost of dedicated equipment, and to integrated unit-dose analytical devices, which can be operated at the point of care [15,21].

Here, we report the adaptation of a light-scattering immunoassay (Unimate®; Roche Diagnostic Products, Welwyn Garden City, UK) for HbA1c to a routine, automated clinical chemistry analyzer (Dimension®; Dade International, Dudingen, Switzerland) and its comparison with a colorimetric method for measuring THb. The immunoassay is based on the release of hemoglobin from erythrocytes, followed by enzymatic digestion with pepsin to release β-N-terminal fragments of hemoglobin; these fragments are then bound to a fixed amount of monoclonal antibody, present in excess. The remaining (unbound) antibody molecules are subsequently reacted with a synthetic polymer (a polyvalent complex of the β-N-terminal fragments) to form an agglutination, the amount of which can be determined by turbidimetry and is inversely related to the concentration of HbA1c. The amount of THb present is obtained from the same hemolysate by using the alkaline hematin method described in the Roche product information. In the absence of an internationally agreed upon reference material, the Roche method applies appropriate correction factors established from a previous evaluation to adjust the results of the light-scattering method to match the results of the HPLC method used by the recent Diabetes Control and Complication Trial [1].

The immunogen used to develop the monoclonal hybridoma cells is a synthetic peptide consisting of the six N-terminal amino acids identical to the human hemoglobin β-chain and glycated on the N-terminal valine residue. As reported elsewhere, the first three amino acid residues, together with the glucose moiety of the β-N-terminal hemoglobin fragment, constitutes the epitope for the monoclonal antibody, which has also been used in another immunoassay method [22]. This offers the potential advantage of a decreased sensitivity to change in HbA1c recognition at the sixth amino acid residue (Glu), the site at which the most common point-mutations occur that give rise to such hemoglobinopathies as HbS (sickle cell) and HbC [23].

Materials and Methods

ADAPTATION OF REAGENTS TO THE DIMENSION ANALYZER

Although the Dimension allows for the measurement of THb before the reagents are added for the HbA1c immunoassay, our final adaptation for HbA1c quantification is based on a two-cuvette approach: THb and HbA1c are assessed separately and the %HbA1c is calculated off-line. %HbA1c may also be obtained as a “calculated result” from updated Dimension software (awaiting implementation at the time this report was prepared).

Effect of variations in the amounts of HbA1c reagents. Signal response at 540 nm was greatest when we doubled all of the reagent volumes specified in the Roche method instructions. Minor adjustments for diluent flush volumes were insignificant; however, decreases of only 20% in the volume of antibody–latex reagent used reduced the signal by 60%.

Reaction linearity and optimal wavelength. We used a fixed-interval rate format to quantify HbA1c. The wavelength recommended by Roche (540 nm) demonstrated a 120-s period of linearity during the progress of the reaction; this measuring interval gave satisfactory increases in signal at both extremes of the HbA1c assay range (30–250 mA). We chose to measure absorbance at 45 and 145 s after the agglutination had been started by addition of reagent R3 (see below).

HbA1c calibration. We serially diluted the Unimate monomeric calibrator, to represent HbA1c concentrations of 0.8–12.0 μmol/L. To construct the calibration curve, we followed an iterative five-parameter logistic curve-fitting procedure, as found on the Dimension, but using a software package (Deltagraph®; Delta Point, Monterey, CA) to calculate the initial coefficients off-line. A typical calibration is shown in Fig. 1.

Effect of incubation time and temperature. A minimum pre-incubation of sample with hematin reagent for 5 min at 37 °C was found to be necessary for absorbance stability across the major peaks of the derivatized form of hemoglobin (“Alkaline Hematin D-575”).

![Fig. 1. HbA1c calibration with five-parameter logistic (logit) curve fit.](https://academic.oup.com/clinchem/article-abstract/43/1/76/5640626/7626)
%HbA1c determinations

By the Dimension analyzer. The Dimension analyzer was obtained from Dade International. Reagents, supplied by Roche under the product name Unimate HbA1c (cat. no. 0755656), were as follows: R1, hematin reagent in 400 mmol/L potassium phosphate buffer, pH 8.1, containing 2 g/L bovine serum albumin and 150 mmol/L sodium chloride; R3, a β-N-terminal fragment polyvalent complex (0.5 mg/L) in 10 mmol/L formate buffer, pH 3.0, also containing 1 g/L bovine serum albumin. Hemolysis reagent consisted of porcine pepsin (>100 kU/L) in 20 mmol/L citric acid. The HbA1c calibrator consisted of a monovalent β-chain-glycated HbA1c peptide (Roche; cat. no. 0755664, stated value 25.6 pmol/L); the THb calibrator (Bayer) had a stated value of 9.1 mmol/L.

The Dimension analyzer can make as many as three additions of reagents or mixtures of individual components plus an aliquot of sample into a cuvette manufactured on board the analyzer. In addition to using the normal printout of results, we captured on computer the raw absorbance (“filter”; see below) data from the defined photometric readings of each method, using a switching device into a Microsoft Excel® spreadsheet by means of an interface communications application (Versaterm Pro®, Synergy Software PCS, Reading, PA). Empty Dimension reagent containers (“flexes”) were punctured to allow introduction of the appropriate reagents for the Dimension open-channel application. The flex configurations were 3.5 mL of R2 in wells 1–5 and 3.5 mL of R3 in well 6 for the HbA1c immunoassay, and 3.8 mL of distilled water in well 1 and 3.8 mL of R1 in wells 2–6 for the THb assay. The reaction for the HbA1c method was started by adding R3 after a 5-min preincubation of sample with R2 (antibody-coated latex); monochromatic readings of absorbance at 540 nm were then performed at 45 and 145 s from this point. The THb assay likewise required a 5-min preincubation of sample and reagent (R1), after which the reaction volume was diluted with distilled water; a single photometric reading (at 405 nm) was performed 1 min later.

By HPLC. Dimension results were compared with an automated HPLC analyzer dedicated to measurement of %HbA1c (Daiichi Hi Auto HA-8140; UK distributor: Biomen Diagnostics, Finchampstead, UK). Glycated forms were separated by reversed-phase ion-exchange chromatography. The desired peaks, defined by retention time, were used to quantify %HbA1c by an on-board calculation algorithm for determining the areas under the curves.

By enzyme immunoassay. We also compared the Dimension results with those of an enzyme immunoassay (Dako Diagnostics, Ely, UK) in which HbA1c is captured and denatured on the surface of a microtitre plate well. The amount of antigen is detected by using an enzyme-labeled monoclonal antibody directed against the glycated N-terminal sequence of the first 8 amino acid residues [14]. The results were related to calibrators of known %HbA1c (assigned by HPLC).

Samples

All blood samples were obtained from routine laboratory operation and had been submitted for determination of HbA1c as part of individual patient management. The samples were collected into EDTA-containing tubes and stored at 4 °C until analysis, all work being completed within 1 week of collection. Samples for the comparisons between methods were drawn from six patient groups: nondiabetics (n = 50), diabetics (n = 50), patients with renal failure (n = 20); urea and creatinine concentrations of 16–33 mmol/L and 318–1150 μmol/L, respectively), patients with hyperlipidemia (n = 10; triglycerides 3–12 mmol/L), patients whose samples were icteric (n = 10; bilirubin >100 μmol/L), and patients with identified hemoglobinopathies (n = 20; HbS and HbC, 10 each).

We also used three in-house control pools of whole blood, prepared to assess defined aspects of method performance. Each pool contained clinically low, medium, or high amounts of HbA1c. To ensure homogeneity of sample, we thoroughly mixed each pool before dividing it into aliquots and storing these frozen at −70 °C ready for use.

Statistical analyses

An ANOVA procedure recommended by the National Committee on Clinical Laboratory Standards [24] was used to estimate individual aspects of imprecision such as total assay and within-assay reproducibility, calibration stability, and on-board reagent stability for both %HbA1c and the separate components THb and HbA1c. Using Microsoft Excel 5.0, we constructed a spreadsheet to perform the ANOVA calculations [24]. Chi-squared test was used to assess the significance of the calculated reproducibilities relative to target values having been previously defined as analytically correct or to identify the significance of factors that contributed to the total imprecision.

Regression analysis performed by the method of Passing and Bablok [25] was used for the method comparison studies; 95% confidence limits for slope and intercept
were calculated from the standard error of regression and used to determine concordance with the target values of 1.0 and 0.00, respectively [26]. This procedure not only offers the advantage of the Deming regression [25], where errors in both $x$ and $y$ planes are taken into account, but also is independent of the distribution of errors in both planes and is not unduly influenced by the presence of extreme points or outliers; i.e., all measurement points have equally valid weighting in the estimation of the regression line.

The statistical package used was Astute (Diagnostic Development Unit, University of Leeds, Leeds, UK). For the other assessments of performance, simple linear regression was used to determine compliance of observed results within defined tolerance limits.

**Evaluation and Validation**

**Imprecision.** Both within-assay and total assay reproducibility were determined by the ANOVA procedure: a one-run-per-day design in which the three in-house pools (low, medium, and high %HbA1c) were analyzed in duplicate over a period of 20 days with use of daily calibration. The within-assay reproducibility was calculated from the average of each day’s mean and SD; the spreadsheet ANOVA calculation provided the total reproducibility of the assay. An alternative estimate of assay imprecision in the form of a sample precision profile was also calculated from the observed variation (CV) in duplicate analyses of the clinical samples ($n = 160$) used for the method comparison studies.

**Stabilities of calibration and on-board reagents.** Calibration stability was determined by reanalyzing each day’s "filter" data obtained from the imprecision data and calculating values according to the calibration from Day 1. The total assay reproducibility was determined by the recommended ANOVA procedure. Stability of the reagents remaining on board the Dimension with punctured flexes required by the Dimension open-channels format was also assessed by the off-line analysis of the captured filter data during the 20 days and calculated from the first day’s calibration.

**Accuracy.** Method comparisons were performed on 160 specimens of whole blood from the patient groups defined previously. Duplicate analyses were performed with the method adapted to the Dimension analyzer, singleton analysis with the HPLC and EIA methods (calibrated where appropriate according to the respective manufacturer’s instructions). Quality-control samples recommended for these methods were regularly assayed in the same runs with the unknowns.

**Sedimentation stability of particle reagent.** The Roche literature recommends an initial mixing of the antibody-coated latex reagent. Concerns about the effect of potential sedimentation of the latex-antibody component over time when loaded onto the Dimension reagent carousel led us to analyze aliquots of control materials (low, medium, and high concentrations) in duplicate every hour for 1 day. Freshly thawed aliquots of control material were used each time, and the reagent was left undisturbed for the duration of the assessment.

**Sample stability.** To assess sample deterioration during storage at 4 °C, we chose at random 10 freshly collected samples and analyzed them serially in duplicate with the Dimension over 17 days.

**Influence of Schiff base and hematocrit.** To determine whether any cross-reactivity ensued from alteration in the Schiff base, we analyzed samples that had been incubated with various concentrations of glucose. A single whole-blood sample with an %HbA1c value within the routine reference range was divided into three aliquots and centrifuged; the resulting plasma was put aside. The three erythrocyte samples were then resuspended in phosphate-buffered saline, 20 mmol/L glucose solution, or 50 mmol/L glucose solution and incubated at 37 °C for 4 h. After centrifugation (<2 min) to remove the incubating solutions, we resuspended the erythrocytes in their own plasma and analyzed them in duplicate for %HbA1c within 15 min after the removal of the incubating solutions.

To determine the effect of hematocrit, we centrifuged a single sample of whole blood for which %HbA1c and hematocrit were within the quoted reference ranges and separated the plasma from the cells. We then recombined the two components in various proportions (10% to 100% erythrocytes, by volume), to create samples with a range of hematocrit values, and analyzed each sample in duplicate.

**Results**

**Imprecision.** Estimates of within-assay and total reproducibility (CV) for the Dimension determinations of %HbA1c were ≤4.0% and ≤6.5%, respectively, for each of the three control pools. Both values conform to target specifications [27], being not significantly different from CV = 5% as confirmed by chi-squared analysis (Table 1). The relation between mean control pool results and duration of storage demonstrated in all cases that the confidence limits calculated from linear regression encompassed a slope of zero. The observed scatter about the line of best fit was random and fell <5% from each pool mean, thereby confirming the absence of any trends during the 20-day period of assessment. Respective slopes and 95% confidence limits for each pool were: low, 0.012 ± 0.029; medium, 0.011 ± 0.031; and high, 0.035 ± 0.039. Sample imprecision calculated from a precision profile gave a mean CV of 4% across the full analytical range of 2–15% HbA1c.
Calibration stability. There was no significant difference (chi-squared analysis) between the imprecision due to calibration for total assay reproducibility (CV < 4.8%) and the CV target of 5% (Table 2). The variation of mean results calculated from the Day 1 calibration during the 20-day assessment period also demonstrated a zero slope within 95% confidence limits and minimal (<5%) random scatter about the regression line. Respective slopes and 95% confidence limits for each were: low, 0.002 ± 0.018; medium, 0.002 ± 0.028; and high, 0.0008 ± 0.042. The calibration was stable for at least 20 working days or one calendar month.

Stability of on-board reagents. Fig. 2 shows the plot of daily means for each pool during the 20-day study, together with results of the linear regression analysis and 95% confidence limits of the slope for each pool provided. The line of best fit dropped below the limits of ± 5% variation about the means of the controls from Day 1 at Day 12 for the medium and high pools. The low pool showed a 10% drop after 3 days; after that, the mean values stabilized. The Day 20 results for the low, medium, and high controls were respectively 12%, 9%, and 0% lower than those on Day 1. Assay reagents on board the Dimension, which were contained within punctured wells, were stable for 1–2 days across the range of %HbA1c.

Method comparison. The comparison data are shown graphically in the form of scattergrams with regression analysis statistics calculated according to Passing and Bablok (see Fig. 3). As evidenced by the concordance of observed slopes and intercepts with respective target values of 1.00 and 0 (within 95% confidence limits), the results from Dimension showed close overall agreement with HPLC; the slope displayed a small but significant rotational bias, 0.902 ± 0.04, but the intercept, −0.096 ± 0.25 was not significantly different from 0. The EIA demonstrated a larger and significant negative bias in

| Table 1. Total and within-assay reproducibilities from low, medium, and high pools for %HbA1c, THb, and HbA1c. |
|---|---|---|---|
| Pool | Mean | SD | CV, % | SD | CV, % |
| %HbA1c | | | | | |
| Low | 5.94 | 0.26 | 4.43 | 0.39 | 6.48 |
| Medium | 7.47 | 0.29 | 3.89 | 0.42 | 5.61 |
| High | 11.90 | 0.47 | 3.94 | 0.58 | 4.89 |
| THb, mmol/L | | | | | |
| Low | 4.72 | 0.16 | 3.37 | 0.43 | 9.23 |
| Medium | 8.75 | 0.28 | 3.25 | 0.66 | 7.49 |
| High | 10.00 | 0.25 | 2.49 | 0.64 | 6.42 |
| HbA1c, μmol/L | | | | | |
| Low | 2.79 | 0.075 | 2.70 | 0.16 | 5.64 |
| Medium | 6.55 | 0.070 | 1.07 | 0.29 | 4.44 |
| High | 11.85 | 0.28 | 2.36 | 0.81 | 6.80 |

* In all cases the chi-squared probability (P) was > 0.05; i.e., the difference between the target CV of 5% and the observed total and within-assay CVs was not significant.

| Table 2. Calibration stability: total and within-assay reproducibilities determined from low, medium, and high pools for %HbA1c, THb, and HbA1c. |
|---|---|---|---|---|---|
| Pool | Mean | SD | CV, % | SD | CV, % |
| %HbA1c | | | | | |
| Low | 6.18 | 0.27 | 4.39 | 0.29 | 4.74 |
| Medium | 7.86 | 0.24 | 3.12 | 0.36 | 4.64 |
| High | 11.95 | 0.43 | 3.63 | 0.60 | 5.00 |
| THb, mmol/L | | | | | |
| Low | 4.70 | 0.15 | 3.23 | 0.30 | 6.36 |
| Medium | 8.62 | 0.27 | 3.12 | 0.55 | 6.41 |
| High | 9.84 | 0.24 | 2.46 | 0.61 | 6.23 |
| HbA1c, μmol/L | | | | | |
| Low | 2.90 | 0.08 | 2.69 | 0.17 | 6.02 |
| Medium | 6.76 | 0.07 | 1.02 | 0.33 | 4.90 |
| High | 11.75 | 0.24 | 2.01 | 0.80 | 6.81 |

* As in Table 1.
The Dimension and HPLC methods showed a positive bias as great as 2.5 times in results for patients with hemoglobinopathies, whereas the EIA method appeared to give no change in %HbA1c in this patient group in comparison with the HPLC method. A separate comparison between all three methods that excluded the results from the patients with renal failure showed essentially no difference in the slopes and intercepts of the regressions from those shown in Fig. 3; i.e., for Dimension vs HPLC, Dimension vs EIA, and EIA vs HPLC, the respective slopes were 0.89, 1.02, and 0.87, and the respective intercepts were −0.01, 0.64, and −0.62. Closely similar results were also observed when the patients with high concentrations of bilirubin or triglycerides were omitted from the comparisons.

**Sample stability.** As Fig. 4 shows, there was no significant change in results over the 17-day period of storage and analysis for any of the 10 samples measured.

**Influence of Schiff base effect and hematocrit.** The samples incubated with glucose at either concentration demonstrated no significant variation of results from that of the samples incubated in phosphate-buffered saline: %HbA1c values were 5.6% in buffer, 5.4% in 20 mmol/L glucose, and 5.8% in 50 mmol/L glucose. There was also no change in %HbA1c values at hematocrits ranging between 30% and 100%; i.e., mean %HbA1c = 4.6% (range 4.5–4.7%). At 10–20% erythrocytes, however, the %HbA1c measured increased to 5.0%.

---

Fig. 2. Scattergram and linear regression between mean results of low (○), medium (■), and high (▲) HbA1c pools and number of days on-board the Dimension analyzer (reflecting reagent stability). Respective slopes ± 95% confidence limits calculated for each pool were: low, −0.039 ± 0.024; medium, −0.032 ± 0.027; and high, −0.055 ± 0.054.

slope (0.863 ± 0.064) and intercept (−0.825 ± −0.268) in comparison with the HPLC assay, but closely agreed with the Dimension assay: slope, 1.024 ± 0.046, and intercept, 0.687 ± 0.21.

<table>
<thead>
<tr>
<th>Method Comparison</th>
<th>Slope (95% CL)</th>
<th>Intercept (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimension vs HPLC</td>
<td>0.902 ± 0.04</td>
<td>0.096 ± 0.26</td>
</tr>
<tr>
<td>Dimension vs EIA</td>
<td>1.024 ± 0.046</td>
<td>0.687 ± 0.21</td>
</tr>
<tr>
<td>EIA vs HPLC</td>
<td>0.863 ± 0.064</td>
<td>−0.825 ± −0.268</td>
</tr>
</tbody>
</table>

Results of hemoglobinopathy samples, depicted by the symbols (○) for HbS and (■) for HbC variants, are overlaid on the scattergrams but excluded from the calculation of coefficients. The dotted lines indicate the envelope of ± 95% confidence limits about the regression line (thick black line).

---

**Fig. 3.** Scattergrams and Passing–Bablok regressions for method comparisons of (top) Dimension vs HPLC (○), (middle) Dimension vs EIA (■), and (bottom) EIA vs HPLC (▲). Respective slopes ± 95% confidence limits calculated for each comparison were (top) 0.902 ± 0.04 and −0.096 ± 0.26, (middle) 1.024 ± 0.046 and 0.687 ± 0.21, and (bottom) 0.863 ± 0.064 and −0.825 ± −0.268. Results of hemoglobinopathy samples, depicted by the symbols (○) for HbS and (■) for HbC variants, are overlaid on the scattergrams but excluded from the calculation of coefficients. The dotted lines indicate the envelope of ± 95% confidence limits about the regression line (thick black line).
Sedimentation stability of particle reagent. Variation in the mean hourly concentrations of R2 over time was assessed by regression analysis. The results (Fig. 5) demonstrate the absence of any significant change of slope during the 1-day period of analysis, within calculated 95% confidence limits.

Discussion

We had originally hoped that the unique features of the Dimension, whereby the absorbance of the reaction can be monitored between addition of reagents, could be utilized to measure both the THb concentration and the inhibition of aggregation by HbA1c in one cuvette. However, the current formulation of the reagents precluded this development, the absorbance of the hemoglobin being insufficient for reliable measurement at any of the 10 available monitoring wavelengths on the Dimension in the presence of the high background light-scattering signal. Therefore, it was necessary to quantify the two components in separate reaction cuvettes.

The within-assay and total reproducibilities of the %HbA1c method, as assessed for imprecision (CV) on the basis of daily calibration, were 4.0% and 4.0–6.5% across the assay range—values comparable with those presented in many reports in the literature for other methods [11, 16, 20]. The imprecision marginally improved to ~3.6% (within-assay reproducibility) and ~4.8% (total reproducibility) when the results were calculated on the basis of the initial day’s calibration, an illustration of the stability of the reagents. This level of imprecision meets the requirements of a total reproducibility of CV 5% identified by the National Diabetes Data Group [27] and is similar to the goal specifications defined by other strategists [28–30]. The reagents have been demonstrated to be stable during a whole day on board the Dimension without mixing, and the stability of samples kept at 4 °C was essentially unchanged throughout a normal working week. A small trend in decreasing %HbA1c was observed when open-well on-board reagents (punctured flexes) were used in the open-channels format for all three in-house control pools. It is unlikely that this small decrease would be clinically significant, however. The total imprecision data demonstrated that this trend is absent if fresh flexes are prepared daily. Given a future situation where flexes have already been sealed at manufacture after the dispensing of reagents, an initial agitation of the flex before introduction on board the Dimension should be sufficient to maintain stability.

There was close agreement between the Dimension and EIA methods for samples containing normal adult hemoglobin (n = 140) and spanning the range of clinical interest (3–15% HbA1c). A small negative bias in slope was seen when the Dimension method was compared with the HPLC assay. In keeping with the practice bias by the manufacturer and other study methods [11], one can, for the reasons stated in the introduction, use an appropriate correction formula to adjust the final calculated result on the Dimension to account for this small negative bias. A small negative bias in the comparison of the established EIA method with HPLC was greater than that between Dimension and HPLC. The Dimension, therefore, generated results intermediate between these two methods, in the concentration range where the amount of scatter about the line of best fit was sufficient to ensure agreement with the EIA method within the calculated 95% confidence limits of slope and intercept.

The data on samples containing abnormal hemoglobins showed an apparent overestimation by the light-scattering method in comparison with the HPLC method,
and suggested that the variants might be discriminated into two groups that give separate and distinctive positive biases. The HbS results obtained with the Dimension are about double (range 2.0–2.5 times) those of the HPLC method, whereas the HbC results lie almost within the upper boundary (range 1.0–1.3 times) of the scatter found in the comparison of normal hemoglobins. The EIA method clearly demonstrates even greater interference from both hemoglobin variant forms. The complete separation of the variant hemoglobin forms by the HPLC method used in this study permitted the direct calculation of results from peak areas, thus achieving a very high level of accuracy—comparable with that of a labor-intensive isoelectric focusing method reported to give the highest specificity [28]. Other dedicated HPLC analyzers, because of incomplete separation, calculate HbA1c by comparison with calibrant chromatograms stored on the instrument [20]. In the absence of a reference method or material, the Daiichi method is arguably one of the best automated HPLC methods and accurately quantifies %HbA1c and its variants. We therefore concluded that the Dimension method gave a better agreement with HPLC results and performance than did the established EIA method.

The monoclonal antibody used in the EIA [14] was raised against the amino acid sequence of the first eight N-terminal residues; the HbS and HbC variant changes at position 6 (of Glu to Val or Lys, respectively) would therefore interfere with antibody recognition. The situation with the antibody used in this study is more complex. After peptic digestion (cleavage of aromatic residues on the N-terminal side), the β-N-terminal fragment, which contains the epitope on the first 3 amino acids linked to the Amadori glucose, is a 14-residue peptide. The variant change at position 6 from a negatively charged and acidic amino acid (resembling HbA) to either a hydrophobic (e.g., HbS) or oppositely charged basic residue (e.g., HbC) will inevitably result in a significant change in the secondary structure of the fragment. The conformational presentation of the epitope to antibody will be altered, which may influence recognition. The observed positive bias between Dimension and HPLC methods leads to the conclusion that the variant change in amino acid may be principally due to enhanced affinity of the antibody–antigen interaction, thereby leaving less latex-bound antibody to agglutinate with the polyvalent antigen reagent. Other, less likely, possibilities may be that the glycated forms of the hemoglobin variants bind the monoclonal antibody nonspecifically under the conditions of the assay, or that the agglutination between unreacted antibody and polyvalent antigen is inhibited by some other mechanism. Studies are currently in progress with HbS, HbC, and other variant forms (such as HbD and HbE) in which the point mutations are much further removed from the N-terminal side; these studies use nondigested hemoglobin chains in which the N-terminal epitope has been exposed by unfolding of the β-chain (the HbF variant does not contain β-chains and so is not included).

A review of the literature on actual performance of HbA1c immunoassays in relation to glycated hemoglobin variants is limited to assessment [18] of the EIA method of Dako [14] and the light-scattering method of the DCA 2000™ [22], developed and manufactured by Bayer. The former does not recognize hemoglobin variants at all; the latter, however, shows results similar to those of affinity chromatography, which correctly estimated all variants except homozygous HbS and HbC. Alternative light-scattering immunoassays, using broadly the same principle as described here, claim to detect variants HbS, C, E, and D through the specificity of the polyclonal antibody, residing in the first four amino acids of the β-chain N-terminus; one such method is part of an automated analyzer, the other is an integrated unit device [16]. To the best of our knowledge, however, assessments of their performance in the literature have not included hemoglobin variants.

Some methods for quantifying HbA1c have been reported to give falsely high results in patients with chronic renal failure [31, 32]; results in such patients are also high for measurements of fructosamine, then decrease after dialysis. The immunoassay for HbA1c described here is not affected by exposure of hemoglobin to higher concentrations of urea or other metabolites. Patients with conditions associated with a shortened lifespan of the erythrocyte (e.g., hemolytic anemia, homozygotes for HbS or HbC) will inevitably invalidate this and all other methods unless new reference ranges can be established [19]. There was no evidence of other interferences in patients with lipemia or hyperbilirubinemia within the conditions assessed. The presence of the Schiff base intermediate and variations in hematocrit, as would be encountered under most clinical circumstances, did not significantly change any result.

In summary, this adaptation offers the user all the benefits provided by an automated analyzer, e.g., rapid sample throughput (up to ~150 tests/h), minimum operator intervention, and a small pretreatment step (hemolysis) performed off-line. The total imprecision of the method is enhanced by the stability of the reagents (reflected in the need to calibrate only about once a month). Improvement in method imprecision by use of stable reagents, thereby reducing the variation implicit in frequent calibration, has been observed for other light-scattering immunoassays [33–35]. We conclude that the light-scattering immunoassay described here provides a rapid, precise, and accurate means for estimating the proportion of HbA1c, the first result being available within 8 min of sample insertion into the analyzer after the hemolysis pretreatment step. As adapted to the Dimension analyzer, the assay shows close agreement with an HPLC/ion-exchange method, which we consider the method currently available that is nearest to being an automated reference procedure. Be-
cause %HbA1c estimates are inappropriately high in the presence of some hemoglobinopathies, it will be important to recognize the presence of variants when reporting results.

P.H. and D.J.N. were funded by a grant from Dade International, Dudingen, Switzerland. We thank Roche Diagnostic Systems, Welwyn Garden City, UK, for their gift of some of the reagents.

References


