The determination of glycohemoglobin [HbA1c, HbA1, or total glycohemoglobin (GHb)] has become an established procedure in the management of diabetes mellitus. Here, we describe the development of a simple, fluorescence, non-separation assay for the percentage of GHb (%GHb). The fluorescence of an eosin-boronic acid derivative when it was mixed with hemolysates of unwashed erythrocytes was quenched in proportion to the percentage of glycohemoglobin. Measurement of the fluorescence intensity gave an estimate of GHb in the sample, and measurement of light absorbance gave an estimate of total hemoglobin. A combination of the two measurements gave the assay response. Comparison with HPLC (Menarini-Arkray HA-8140 fully automated analyzer) for the percentage of HbA1 (%HbA1) gave %GHb(NETRIA) = 1.1(SD ± 0.03)%HbA1 + 0.6(SD ± 0.3), S_{GHb} = 0.821, r = 0.972, n = 80; comparison for HbA1c gave %GHb(NETRIA) = 1.3(SD ± 0.04)%HbA1c + 1.8(SD ± 0.3), S_{GHb} = 0.813, r = 0.973, n = 80. Precision, estimated as the percentage of the CV of the %GHb assay results, was <2% (intraassay, range 5–22% GHb) and <4.2% (interassay, range 4–16% GHb). Dilution of a high-percentage GHb sample lysate showed that the assay was linear, and addition of glucose (60 mmol/L), bilirubin (250 μmol/L), and triglycerides (14 mmol/L) to low, medium, and high %GHb samples showed no clinical interference in assay results.

Glycohemoglobin has become the established method for assessing long-term glycemic control for diabetic patients (1). A percentage of hemoglobin becomes glycated during the lifetime of erythrocytes (120 days), reflecting the integrated mean blood concentration of glucose over the preceding 8–12 weeks (2, 3). There is a wide range of methods for the determination of glycohemoglobin [HbA1c, HbA1, or total glycohemoglobin (GHb)], and they have been reviewed recently (1, 4). Glycohemoglobin is made up of a number of chemically distinct species, which are produced by the binding of glucose and other carbohydrates to various sites on hemoglobin. HbA1c is glucose bound via Amadori rearrangement to the N-terminal valine of the β chain. HbA1 is composed of all the carbohydrate species, including glucose, derived from adducts bound to the N-terminal valine. GHb is the total of all carbohydrate derivatives bound anywhere on the hemoglobin molecule. To date, no clinical advantage has been shown for measuring HbA1c as opposed to HbA1 or GHb (1).

Here, we present the development of a non-separation method for the determination of GHb. The principle on which the method is based is that the emissions of certain fluorescent derivatives of boronic acids are quenched when bound to glycohemoglobin. In previous publications, the quenching of a fluorescein-boronic acid derivative was described (5, 6). The fluorophore showed fluorescence quenching when mixed with purified glycohemoglobin but not with non-glycohemoglobin at similar concentrations. Addition of the fluorophore to erythrocyte lysates gave quenching values that correlated well with the electroendosmosis method for HbA1 (Corning Glytrac; r = 0.91, n = 25), and gave valid results with three heterozygous HbA/S samples. It was considered that the fluorescence quenching method could be improved by the selection of a fluorophore whose emission gave a high degree of spectral overlap with an absorption band of hemoglobin. This overlap would, it was predicted, lead to enhanced quenching of the fluorophore when bound to glycohemoglobin. The emission spectra of an eosin-boronic acid derivative gave the required spectral characteristics, with emission coincident with the 540 nm absorption band from hemoglobin (Fig. 1). We describe results using the eosin-boronic acid derivative and the development of an assay for GHb.

Materials and Methods

EOSIN-BORONIC ACID DERIVATIVE

An eosin-boronic acid derivative of eosin-5-isothiocyanate and 3-aminophenyl boronic acid was prepared by the method described previously (6). An aqueous solution
was made and stored at 4 °C. Assays were performed over an 11-month period from one batch of reagent, which showed no signs of degradation. Other batches have also been prepared and gave close agreement in tests.

ASSAY BUFFER
The buffer was composed of 100 mmol/L glycine (AnalaR; BDH) and 50 mmol/L magnesium chloride hexahydrate (AnalaR; BDH), pH 8.5 (pH adjusted to 8.5 by addition of saturated sodium hydroxide).

SAMPLES
Aliquots were taken from clinical samples (EDTA whole blood) taken for routine clinical analysis. All measurements were performed within 7 days of the sample being taken. None of the samples gave evidence of abnormal hemoglobins by the HA-8140 HPLC method.

CALIBRANTS
Lyophilized red blood cell lysates (Menarini) were reconstituted in distilled water to give an absorbance (405 nm) within the correction range of the assay (Fig. 4).

FLUOROMETER AND SPECTROPHOTOMETERS
The fluorometer was a Perkin–Elmer LS50B adjusted to the following settings: excitation, 523 nm; emission, 542 nm; slits, 5 nm/5 nm; and integration time, 5 s. All intensity values were on an arbitrary scale. The spectrophotometers were a Cecil Instruments 5233 and Anthos HT II, equipped with a 405 nm filter.

HPLC METHOD
The HPLC was an Menarini-Arkray HA-8140 fully automated analyzer (Arkray, formerly KDK) (7). Results were given as percentages of HbA1c, HbA1, and HbF.

ASSAY DEVELOPMENT
Concentration of reagents. The concentrations of the eosin-boronic acid derivative and red cell lysates were adjusted to give optimum discrimination between low and high percentage GHb (%GHb) samples.

Time of reaction. A sample lysate-fluorophore mixture was prepared according to the assay method in a square 1-cm glass cuvette (four clear sides) sited in the fluorometer. On addition of the eosin-boronic acid solution to the lysate, continuous measurement of the fluorescence intensity was begun and was monitored for 800 s. A graph of fluorescence intensity against time was plotted (Fig. 2).

Fluorescence quenching results. The fluorescence intensity of the eosin-boronic acid solution with and without sample lysates allowed calculation of the percentage of quenching to be made. Subtraction of the background gave the quenching index (QI). Comparison of fluorescence QI results with the percentage of HbA1 (%HbA1) showed some agreement (r = 0.81, n = 34, Fig. 3). The samples showing positive deviation from the %HbA1 result were all found to have higher absorbances than those that showed negative deviation.

Correction of quenching results estimated by absorbance. The relationship between absorbance (405 nm) and quenching correction (8QI) was obtained from experiments where the difference between QI results and %HbA1 results were plotted vs absorbance. Tests were carried out for individual samples at different concentrations and for different samples at different concentrations. Investigations of the relationship between absorbance (405 nm) and 8QI for samples at different concentrations (absorbances 0.37–0.52) gave a correlation coefficient of 0.98 (n = 20) and a slope of 55.7 (linear regression). Samples at different

![Fig. 1. Absorption spectrum of hemoglobin and emission spectra of a fluorescein-boronic acid (FBA) (5, 6) and the eosin-boronic acid derivative (EBA).](image1)

![Fig. 2. Time course for reaction between sample lysate and eosin-boronic acid.](image2)
Concentrations were found to lie close to this slope; therefore, the slope of the regression relationship, shown in Fig. 4, was adopted with minor modification to the intercept for use in assays. Application of $\delta QI$ to the quenching data gave the assay response as the corrected quenching index ($QI_{corr}$, Fig. 5).

**Calibration.** Assay responses ($QI_{corr}$) gave a significant correlation with %HbA$_1$ ($r = 0.972$). A Bland-Altman plot (Fig. 5) (8) showed that a linear method of calibration would be appropriate. The lyophilized red cell lysates described above allowed linear calibration of assays with %GHb values assigned (5.0% and 14.5%). These values were assigned to the lyophilized pools to give a range of %GHb values that gave good agreement with the HPLC method.

**Assay method.** A dilute solution of the eosin-boronic acid derivative was prepared from stock in the assay buffer containing detergent. This was prepared immediately before use in each assay.

One milliliter of whole blood (EDTA) was pipetted into a 5-mL test tube and centrifuged at 173g or allowed to settle overnight. Fifty microliters of the packed erythrocyte layer were added to 400 µL of lysing buffer (detergent solution in assay buffer), gently mixed by hand until homogeneous, and allowed to stand for a minimum of 10 min.

Twenty microliters of lysate or reconstituted calibrant were mixed with 4000 µL of the eosin-boronic acid solution and left in the dark for 20 min out of direct light, and the fluorescence intensity was read ($\lambda_{ex} 523$ nm/$\lambda_{em} 542$ nm, details above). The absorbance of each sample was determined by pipetting 200 µL of each sample-fluorophore mixture into microtiter wells and measuring the absorbance at 405 nm on a spectrophotometer (see below).

**Assay method summary.** Following is a summary of the assay:

(a) One milliliter of EDTA whole-blood sample was centrifuged (173g) or allowed to settle overnight.

(b) Fifty microliters of packed red cells were added to 400 µL of lysing buffer, mixed gently, and allowed to sit for 10 min.

(c) Twenty microliters of lysate or reconstituted calibrant were mixed with 4000 µL eosin-boronic acid solution and left in the dark for 20 min.

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**Fig. 3.** Quenching results ($QI$), before correction for hemoglobin concentration, plotted vs %HbA$_1$ (HA-8140).

$r = 0.81$, $n = 34$; $QI_{NETRIA} = 0.99(\%HbA_1) + 0.19$.

**Fig. 4.** Relationship between absorbance (405 nm) and correction applied to fluorescence-quenching results ($\delta QI$).

**Fig. 5.** Quenching results corrected for hemoglobin concentration to give an assay response, $QI_{corr}$, plotted vs %HbA$_1$ (HA-8140).

$r = 0.97$, $n = 34$; $QI_{corr, NETRIA} = 0.99(\%HbA_1) + 1.6$. **
Fluorescence intensity ($\lambda_{ex}$, 523 nm/$\lambda_{em}$, 542 nm) and absorbance (405 nm) were read.

**Calculation of Results**

*Data from assay.* The data from the assay were calculated using the following variables: $T$, fluorescence intensity of the total solution (eosin-boronic acid solution with no sample or calibrant); $F$, fluorescence intensity of the eosin-boronic acid solution with calibrants or samples; and *absorbance 405 nm*, the absorbance of a sample at 405 nm, measured with a 5-mm pathlength.

The percentage of quenching was calculated as $\left[\frac{(T - F)}{T}\right] \times 100\%$. Subtraction of background gives the QI. The results were corrected for hemoglobin concentration (using the *absorbance 405 nm*) to give an assay response. Values for %GHb were obtained for samples by linear interpolation from calibrant responses.

*Intraassay precision.* All sample lysates were measured in duplicate, and a precision profile was plotted (CV values vs mean result; Fig. 9). The curve fit (quadratic) was plotted.

*Interassay precision.* Four assays were performed using separate assay reagents on frozen lysates from patient samples ($n = 6$) stored at $-40^\circ C$, and a precision profile was plotted (CV values vs mean result; Fig. 10). The curve fit (quadratic) was plotted.

*Linearity.* Lysate from a high-percentage GHB sample (20.5% GHB) was diluted in lysate from a low-percentage GHB sample (8.7% GHB). The lysate mixtures (four dilutions and the high and low lysates) were measured according to the assay protocol. The assay results for each dilution (duplicates) were plotted vs the calculated %GHB for the dilutions (Fig. 11).

**Effect of Glucose**

Three whole-blood samples (low, medium, and high %GHB) were each divided into two aliquots. To one aliquot, 9 g/L NaCl was added, and to the other a high concentration of d-(+)-glucose (Sigma) was added to give a final concentration of $\sim 60$ mmol/L. Glucose was measured for both aliquots of blood by the hexokinase method (on an automated analyzer, ILab 900, Instrumentation Laboratories). The two aliquots were also assayed for %GHB, according to the protocol. Results from the samples with added glucose were compared with those for the sample without added glucose and expressed as a percentage (Table 1).

**Effect of Bilirubin**

The method for bilirubin was similar to the method for glucose, with the final concentration of added bilirubin (mixed isomers, Sigma) measured by the serum total bilirubin method (modified Jendrassik-Grof assay on automated analyzer ILab 900). The results are shown in Table 2.

**Effect of Triglycerides**

The method for triglycerides was similar to the method for glucose, with the final concentration of triglycerides (Intralipid, Pharmacia) measured by the serum triglyceride method (automated assay on an automated analyzer ILab 900). The results are shown in Table 3.

**Correlations and Regressions**

The correlations and regressions were calculated according to the method of Pearson.

**Results**

Optimization of the concentrations of sample and the eosin-boronic acid derivative gave a quenching-percentage range (after subtraction of background) between 5% and 20% for samples with similar %HbA1 values.

Fluorescence intensity with time measurements of a sample lysate and eosin-boronic acid mixture showed the reaction reached steady-state after 7 min (Fig. 2).

Data from the assay with 34 samples were used to

<table>
<thead>
<tr>
<th>Table 1. The effect of glucose addition.</th>
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<tr>
<td>Glucose, mmol/L</td>
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</tr>
<tr>
<td>0.9</td>
</tr>
<tr>
<td>65.0</td>
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*a Sample very hemolyzed, which gives higher bilirubin values in the assay than are actually present in the sample.

<table>
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</tr>
<tr>
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<table>
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<tr>
<th>Table 3. The effect of triglyceride addition.</th>
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<tr>
<td>Triglycerides, mmol/L</td>
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<td>------------------------</td>
</tr>
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<tr>
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show the fluorescence quenching results before and after correction for hemoglobin concentration (absorbance 405 nm). The uncorrected quenching results for 34 samples (Fig. 3) showed some agreement with the %HbA1 results \((r = 0.81)\). The samples showing positive deviation from the %HbA1 result were all found to have higher absorbances than those that showed negative deviation. Application of the correction calculated from absorbance \((\delta QI,\text{Fig. 4};\text{see the section concerning assay development in Materials and Methods})\) to the quenching data gave the assay response (Fig. 5). These results gave a significant correlation with %HbA1 \((r = 0.97)\). A Bland-Altman plot \((8)\) (Fig. 6) showed that a linear method of calibration would be appropriate. Lyophilized pools of red blood cell lysates with high and low %GHb were found have stable absorbances (405 nm) in the assay and were used as calibrants. %GHb values of 5.0% and 14.5% were assigned to the pools (see description of assay development in Materials and Methods). Sample results calibrated as %GHb gave a slope of 1.1 (intercept 0.6) compared with %HbA1 results.

The performance of the developed method was evaluated by comparison with the Menarini-Arkray (HA-8140) HPLC (7).

A total of 80 samples were measured in duplicate: 34 in one analytical run and 46 in another. The values for %GHb were plotted vs %HbA1 (Fig. 7) and %HbA1c (Fig. 8). Regression and correlation results were as follows: %GHb(NETRIA) = \(1.1(\text{SD} \pm 0.03)\) %HbA1 + 0.6(\text{SD} \pm 0.3), \(S_{\delta y} = 0.821, r = 0.972, n = 80 (34 + 46); \%GHb(NETRIA) = 1.3(\text{SD} \pm 0.04)\) %HbA1c + 1.8(\text{SD} \pm 0.3), \(S_{\delta y} = 0.813, r = 0.973, n = 80 (34 + 46).\)

The intraassay precision (CV, duplicate results plotted vs mean %GHb result, \(n = 80\)) for sample values of 5–22% GHb was <2% (Fig. 9). The interassay precision for six samples assayed four times was <4.2% for sample values of 4–16% GHb (Fig. 10).

The assay showed linearity of response with a high %GHb sample diluted in a low %GHb sample (Fig. 11). Glucose up to a concentration of ~60 mmol/L was found to have no clinical interference on the assay for low, medium, and high %GHb samples (Table 1). Bilirubin up to a concentration of 250 \(\mu\)mol/L showed no clinical interference on the assay for low, medium, and high %GHb samples (Table 2). Triglycerides up to a concentration of 14 mmol/L showed no clinical interference for low, medium, and high %GHb samples (Table 3).

**Discussion**

The results from measuring the quenching of the eosin-boronic acid derivative by hemolysates and correcting for
the hemoglobin concentration of the samples demonstrate a novel assay for glycohemoglobin.

Hemoglobin is known to quench the fluorescence of associated and covalently bound fluorophores (9–12). The eosin-boronic acid derivative has a fluorescence emission spectrum that peaks at 541 nm. This peak is coincident with one of the absorption peaks of hemoglobin (540 nm, Fig. 1). It may be postulated that the covalently bound, excited eosin-boronic acid loses energy by resonance transfer to glycohemoglobin in a manner similar to other fluorophores bound to the β93 Cys residue on hemoglobin (10). The resonance transfer mechanism only occurs for donor-acceptor pairs in close proximity (<10–12 nm) with a high degree of spectral overlap (13). Hemoglobin is 6.4 × 5.5 × 5.0 nm in size; therefore, covalently bound eosin will be in close proximity to one of the four heme units (14). This quenching process forms the specific component of the quenching observed and depends on the amount of glycohemoglobin in the sample covalently bound to the eosin derivative. In addition to this quenching, the presence of hemoglobin in solution with an eosin derivative (not bound) quenches the signal by “inner filter effect” (i.e., the emitted light from the eosin derivative is reabsorbed by hemoglobin) (10). This component of the quenching depends on the absorbance of the sample. Therefore, it can be estimated (Fig. 4) and used to correct the quenching results (Fig. 3, uncorrected; Fig. 5, corrected for hemoglobin concentration). The value of the corrected results (QIcor) was the assay response, and linear interpolation from calibrant responses gave %GHb results.

In contrast to this non-separation method, fluorescence quenching has been used to estimate glycohemoglobin in a separation assay developed for the Abbott IMx™ automated analyzer (9). The method separates glycated and nonglycated hemoglobin and estimates the concentrations by the fluorescence quenching of 4-methylumbelliferone. The interaction between the fluorophore and hemoglobin is thought to be a noncovalent association.

The use of calibrants was considered necessary to ensure maximum consistency between assays. The validity of linear calibration for this method is demonstrated by the Bland-Altman plot (Fig. 6). Calibration for %GHb is the most appropriate for this method because boronic acids bind all cis-diol-containing adducts bound to hemoglobin. The values assigned to the calibrants gave an appropriate range of values for %GHb. Final calibration of this method (as for others) is not yet possible until an international standard becomes available. The method could, if desired, be calibrated with %HbA1c or %HbA1c.
The assay gave a linear response with dilutions of a high-%GHB sample lysate, demonstrating the the validity of the calibration and method of calculating assay response (Fig. 11). The close correlations with the Menarini-Arkray HA-8140 HPLC method demonstrate that this method can accurately determine glycohemoglobin concentrations for diabetic management (Figs. 7 and 8).

The precision results of <2% (intraassay, Fig. 9) and 4.2% (interassay, Fig. 10) are well below the specified target of 5% (intra- and interassay) for glycohemoglobin assays (1). These precision results are an improvement on the manual boronic acid column method (within-batch CV, 3–5%; between-day CV, 3–8%) (15).

Glucose up to a concentration of 60 mmol/L showed no clinical interference in the assay. This finding is similar to those with other methods using boronic acids (9, 16). Bilirubin, a fluorescent compound, may be expected to interfere in a fluorescence non-separation method. However, bilirubin gives negligible fluorescence emission at 542 nm when excited at 523 nm (the wavelengths used in the assay), and results show that it does not clinically interfere with the assay. Triglyceride concentrations up to 14 mmol/L did not clinically interfere with the assay.

Additional studies are required to assess whether abnormal hemoglobin samples are measured equipotent with the HbA samples and whether anemia affects the clinical interpretation of results from this assay. The majority of methods for measuring glycohemoglobin involve a separation step, which increases the technical complexity (9, 16–24). This non-separation method appears to have the advantage of simplicity without compromising precision and accuracy. Furthermore, this non-separation methodology demonstrates a lack of interference from glucose, bilirubin, and triglycerides. Future developments include the manufacture of a purpose-built combined fluorescence and absorption spectrophotometer and the substitution of packed red cell lysates with whole-blood lysates. The method has the potential to be inexpensive and capable of high sample throughput.

We thank Graham Hitman, Garry John, and Alan Williams at the Royal London Hospital, Whitechapel, London, for help and advice. Aspects of the method and principles described in this paper are the subject of an international patent application (6).

References