Evaluation of a new semi-automated high-performance liquid chromatography method for glycosylated haemoglobin

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Introduction

The measurement of glycosylated haemoglobin in blood is vital for monitoring metabolic control in diabetics [1–3]. The development of many different methods of measurement (based on cation-exchange chromatography, electrophoresis, high performance liquid chromatography [HPLC], affinity chromatography, radio and colorimetric immunotechniques) proves the importance of this assay [4]. Since the first HPLC method was described [5] a number of modifications have been reported (see the review by Ellis et al. [6]). Instruments have been specially designed and commercialized (for example by Helena Laboratories, Beaumont, Texas, USA and Kyoto Daichi, Japan).

The evaluation of a recently commercialized semi-automatic HPLC instrument, which performs analyses on hemolysates from the original blood samples, is reported.

Materials and methods

The instrument, commercialized by Bio-Rad Laboratories (Segrate, Milano, Italy), consists of a main unit, containing the functional parts (autosampler, buffer reservoir, pumps, column colorimeter, and a control unit). A single piston pump is used with a step-gradient valve system, by which the elution of the column is performed with three phosphate buffers of pH 5.7, 5.8 and 5.9 (unstated concentration) and increasing ionic strength. The analytical column (4.0 × 150 mm) is filled by a new cation exchanger (TSK gel, unstated composition). The column temperature is controlled at 23°C by forced ventilation in the column compartment.

The sample (5 μl of venous blood, with 1 mg/ml of EDTA) is diluted automatically with 1 ml of haemolysing reagent (0.1% vol/vol polyoxyethylene-ether in borate buffer); 20 μl of the haemolysate are used for analysis. Spectrophotometric detection is performed at two wavelengths (415 nm and 690 nm) to ensure a stable base-line. All the operations are controlled by a microprocessor and a built-in integrator performs data reduction. The final results are printed out together with a chromatogram of the run. The order of elution of the haemoglobin fractions is: HbA_{1a}, HbA_{1b}, HbF, HbA_{1c}, HbA_{o}. Each run takes 8 min.

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Figure 1. Chromatograms of a normal (a) and a diabetic (b) sample obtained after a standard run. X-axis: time (min); y-axis: concentration, as a percentage of the total haemoglobin (upper scale: 10%). The fractions separated are: (1) non-haemoglobin peak; (2) HbA_{1a}; (3) HbA_{1b}; (4) HbF; (5) HbA_{1c}; and (6) HbA_{o}.
The reference method used for the evaluation was the Bio-Rad Laboratories microchromatographic method for HbA1c, performed at 23 °C, as previously described [7]. The alkali denaturation method, modified according to Molden [8], was used for HbF identification.

Blood samples used were collected by the Clinical Chemistry Laboratory and the Clinical Medicine Division (H. S. Raffaele, Milano, Italy). A Coulter S-Plus IV (Kontron, Switzerland) was used to measure the haematological parameters. Some ethylene glycol stabilized haemolysates were also prepared, according to a previously reported procedure [9], and used within two months.

Analytical imprecision was measured using blood samples and a lyophylised control material (Lypocheck Hemoglobin Alc, from Bio-Rad Laboratories).

**Results and discussion**

Figure 1 shows the chromatograms obtained after the analysis of a normal (a) and a diabetic sample (b). The analyser separates four or five haemoglobin fractions depending on the HbF level (see later). The amount of each species and the order of elution are closely similar to those obtained by liquid chromatography with the Bio-Rex 70, under the conditions reported by Wacks et al. [10]. In order to test whether or not the amount of haemoglobin loaded to the column would interfere with analytical accuracy, as reported for the microchromatographic methods [11], samples with different haematocrit values and similar glycosylated haemoglobin levels were analysed. The samples were prepared by diluting packed red blood cells with different volumes of their own plasma. The results obtained showed that, for haematocrits between 22% and 75%, no appreciable effect is produced on the results of analysis, i.e. no haematocrit-effect can be detected.

The analytical precision, within and between runs, was also analysed; the results obtained are reported in table 1.

In order to evaluate the within-series precision the analyses were repeated eight times on a blood sample from a normal subject, 10 times on blood from a diabetic patient and 17 times on the control lyophylised material.

![Figure 2. Comparison with the reference method in the HbA1c determination. The equation derived from the linear regression analysis is: y = 0.991 x + 0.32; n = 43; r = 0.985; P < 0.001. The dashed line correspond to the theoretical regression equation: y = 1.000 x + 0.00.](image)

![Figure 3. Comparison between the present method and the alkali denaturation method, for the measurement of HbF (y = 0.41 x + 0.12; n = 29; r = 0.634; P < 0.001). Dashed line as in figure 2.](image)

**Table 1. Precision.** For each set of data the mean and the standard deviation (as a percentage of the total haemoglobin) are reported. Coefficients of variation are shown in parentheses.

| Sample                  | n | HbA1a (%) | HbA1b (%) | HbF (%) | HbA1c (%) |
|-------------------------|---|-----------|-----------|---------|-----------|
| **Within-run**          |   |           |           |         |           |
| Normal                  | 8 | 0.3 ± 0.1 (2.0) | 0.5 ± 0.1 (2.0) | 0.5 ± 0.1 (7.2) | 4.1 ± 0.1 (1.1) |
| Diabetic                | 10| 0.4 ± 0.1 (3.0) | 0.6 ± 0.1 (5.2) | —       | 6.8 ± 0.1 (1.0) |
| Lypocheck A1c           | 17| 1.1 ± 0.1 (4.2) | 1.9 ± 0.1 (3.0) | —       | 7.3 ± 0.1 (1.5) |
| **Between-run**         |   |           |           |         |           |
| Low-level ethylene glycol haemolysate | 9 | 0.3 ± 0.1 (1.7) | 0.6 ± 0.0 (0.0) | —       | 4.6 ± 0.1 (2.5) |
| High-level ethylene glycol haemolysate | 9 | 0.3 ± 0.1 (8.1) | 0.6 ± 0.1 (4.0) | —       | 7.3 ± 0.1 (1.4) |
Figure 4. Chromatograms obtained from the analysis of haemolysates from subjects suffering from different haemoglobinopathies: (a) normal control; (b) HbS (β6 glu → val) trait; (c) HbE (β26 glu → lys) trait; (d) HbJ Paris (α12 ala → asp) trait; and (e) Hb Lepore trait (8β → fusion). The concentrations of mutant haemoglobins in each sample, determined by cellulose acetate electrophoresis at pH 8.4, with respect to the total haemoglobin concentration were: HbS = 38%; HbE = 33%; HbJ Paris = 24%; and Hb Lepore = 12%.

The between-run precision was tested over a period of one month, analysing two ethyleneglycol stabilized haemolysates nine times (every third day). Each daily measurement was performed in duplicate.

The accuracy in the measurement of HbA1c was evaluated by measuring this fraction in 41 blood samples with both the present method and the reference method. The data obtained are shown in figure 2.
The accuracy of HbF measurement was determined on a limited number of samples, the data being reported in figure 3. It can be seen that the accuracy is not satisfactory. Further methodological improvements must be introduced to resolve this quantitatively minor fraction which has great clinical relevance for the characterization of the β-thalassemia syndromes.

Interference by abnormal haemoglobins was investigated by analysing haemolysates from subjects suffering from various haemoglobinopathies, which had previously been characterized. Figure 4 shows that in all the specimens analysed, the instrument produced an abnormal chromatogram; if an abnormal haemoglobin was present it showed as an anomalous peak, indicated as P4, P5 or P6.

In the case with HbS (see figure 4[a]), the HbA0 peak is split into two fractions, the last one (P6) probably corresponding to the HbS present in the sample. There is good correlation between the amount of this fraction (40-7%) and the HbS concentration in the sample (38%) determined separately by cellulose acetate electrophoresis (CAE) at pH 8.4. The position of the peak suggests also that the fraction may have an electrophoretic mobility slower than that of HbA0, as is to be expected for HbS.

A somewhat similar comparison holds for the sample showing the HbJ Paris trait (see figure 4[d]). This haemoglobin variant has greater mobility than HbA0 on CAE, and it is therefore expected to be eluted among the first fractions. The chromatogram shows an elevation in the HbA1a and HbA1b fractions, which are eluted together. The concentration of these fractions (13.1%) is too high even for a sample from a diabetic person, and indicates the presence of interference. The concentration found using this analysis is significantly lower than that measured by CAE.

In the case of the Hb Lepore trait (see figure 4[e]), a variant with electrophoretic mobility close to that of HbF, an abnormal chromatographic pattern is shown, but detection of the type of the variant from the chromatogram is very uncertain.

Characterization is also impossible in the case of the sample showing the HbE trait (see figure 4[c]). This mutant has a very low electrophoretic mobility at pH 8.4 (similar to that of HbA2) and elution is therefore expected after HbA0. In this case the HbA0 peak is not split, as with the HbS trait sample, but the chromatogram is abnormal at the boundary between the HbA0 peak (elution time = 5.3 min). The final peak shows a small shoulder on its right-hand side.

From our experience we can therefore conclude that the instrument is more sensitive to the presence of abnormal haemoglobins in the sample than are the minicolumns which are normally used. The operator should be alerted by the appearance of anomalous peaks but further electrophoretic investigations need to be performed from full diagnosis. The estimation of the true HbA1c levels can be attempted by a careful observation of the chromatogram, knowing that, under standard conditions, the elution time for HbA1c is about 4.8 min.

The influence of so-called ‘labile glycosylated haemoglobin’ fractions on this method was also examined. Erythrocytes were incubated separately with glucose (40 g/l) and with saline solution (9.0 g/l NaCl) at 37°C, for up to 24 h. No significant increase in the HbA1c levels
of three blood samples (one from normal and two from diabetic subjects) after 24 h of incubation with the glucose solution was detected (see figure 5[a]). Similarly, no significant decrease in the initial HbA1c levels was found in two blood samples (one normal and one diabetic) incubated with saline for 24 h (see figure 5[b]). Furthermore, in order to evaluate the presence of the labile fractions in the incubated erythrocytes, the same samples were analysed for HbA1c levels, using a haemolysing reagent without borate (the same haemolysing reagent previously commercialized Bio-Rad Laboratories for assays of HbA1). This kind of reagent does not eliminate the aldime forms during the haemolysis step. In fact, the results of these determinations (see the dashed lines in figure 5) showed an increase in the HbA1c levels after the glucose incubations, and a significant decrease in the HbA1c concentrations after the incubations with saline.

It can therefore be concluded that the method described here is insensitive to the presence of the ‘labile fractions’, which are reported to interfere strongly with many chromatographic methods.

In this evaluation no experiment was performed to test the effect of temperature on the analytical resolution of the minor haemoglobin fractions. Reports in the literature indicate that this effect is evident with all the procedures based on ion-exchange chromatography and, to a smaller extent, with those using affinity chromatography [12]. It is assumed that a thermoregulated column compartment within the apparatus will eliminate any interference from temperature variations on the chromatographic pattern.

In summary, it would appear that this system could be of significant value for the measurement of glycosylated haemoglobin. It combines good precision and accuracy with practicality for speed, technical skill requirements and safety. The system can also be useful in evaluating samples from subjects suffering from some haemoglobinopathies where diagnosis may frequently be missed by conventional chromatographic techniques, normally used to measure glycosylated haemoglobin levels.

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