

An evaluation of the Diamat HPLC analyser for simultaneous determination of haemoglobins A₂ and F

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The authors describe a modification of the instrumental parameters of the Diamat fully automated HPLC system for Hb A₂ assay (Bio-Rad Laboratories, Milan, Italy) in order to obtain simultaneous determination of Hb A₂ and Hb F.

Hb A₂ and Hb F measurements are reproducible (within-run CV 2.6%, with Hb A₂ 2.7%; 5.1%, with Hb F 1.3%) and accurate (from a comparison with two microchromatographic techniques for Hb A₂: $r = 0.9639$ and 0.9755 ; with two alkali denaturation procedures for Hb F: $r = 0.9990$ and 0.9952 ; with radial immunodiffusion, $r = 0.9877$). Assay linearity has been confirmed for Hb A₂ concentrations between 0 and 6.0%, and for Hb F between 0 and 60%. The data obtained from the analysis of some pathological samples for Hb Bart's, Hb H, Hb J Sardegna, Hb Lepore and Hb S are in agreement with cellulose acetate electrophoresis analysis.

The Hb A₂ reference intervals for normals (N = 597) and Beta-thalassaemia carriers (N = 200) are respectively (95% limits) 2.02–3.27 and 3.92–5.90 in % units. Hb F values measured in normals (N = 968), in β -thal carriers (N = 302) and in $\delta\beta$ -thal carriers (N = 3) have been found to be consistent with the usual diagnostic parameters.

Some minor limitations emerged: the most relevant concerns Hb A_{1c}, which is overestimated with respect to a reference method ($y = 1.217x + 0.16$; N = 79; $r = 0.9235$). A probable interference from labile fractions is responsible for this Hb A_{1c} inaccuracy.

Introduction

The determination of the minor haemoglobins, Hb A₂ and Hb F, in whole blood are of considerable diagnostic use for the characterization of several thalassaemic syndromes and for the correct diagnosis of several haemoglobinopathies [1]. It is also known that elevated

levels of Hb F can be associated with neoplastic conditions, anaemias and leukaemias of different etiologies [2]. Therefore, a variety of laboratory techniques have been developed to accurately measure these minor haemoglobins. The most commonly used are ion-exchange chromatography [3], on microcolumns or with HPLC [4–7], for Hb A₂, and radial immunodiffusion [8] and alkali denaturation [9–11] for Hb F.

This report presents the authors' experience with a new HPLC method performing a simultaneous determination of Hb A₂ and Hb F. This is obtained by using a fully automated HPLC analyser (Diamat, Bio-Rad Laboratories, Segrate, Milan, Italy), originally dedicated to the analysis of glycosylated haemoglobins [12–14], and later adapted to Hb A₂ determination [15].

By introducing a slight modification to the buffer elution times recommended by the manufacturer for Hb A₂ determination, the method was adapted for measuring Hb A₂ and Hb F simultaneously.

The data reported here confirm the utility of the technique to haematological practice. Some minor limitations are also discussed.

Materials and methods

Samples

Whole blood samples (anticoagulated with potassium EDTA, 1 g/l) were selected from routine laboratory samples for most of the analytical tests. Informed consent was obtained from those subjects studied for the evaluation of reference values.

Stabilized Hb A₂ and Hb F liquid control materials were obtained by adding ethylene glycol (0.35 volume fraction) to haemolysates prepared by a standard tetrachloromethane lysis procedure [16]. These solutions total Hb 100 ± 20 g/l were stored at -20°C and used for long-term precision estimates. This was carried out by assaying two haemolysates, with low and high Hb A₂ and Hb F concentrations, over periods of about one month (the Hb A₂ test) and three months (the Hb F test).

Procedures

Diamat HPLC analysis

This method basically consists of a cation-exchange liquid chromatography developed with three tris-phosphate buffers of increasing ionic strength at a

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controlled temperature of 23 °C. The eluate is read at 415 and 690 nm. For the analysis five µl of whole blood are diluted, by means of the dilutor supplied with the apparatus, with 1 ml of haemolizing reagent. The haemolysate is centrifuged at 10 000 *g* for 1 min and is then loaded into the sample compartment, which is kept at between 2 and 8 °C. Twenty µl are injected into the column for each run.

Figure 1 shows the modifications made to the instrumental parameters recommended by the manufacturer in order to obtain a resolution of Hb F from Hb A_{1c}. The best Hb F isolation is achieved by prolonging the buffer 1 elution to 2 min (figure 1[c]); there is no effect if the elution of buffer 2 is prolonged from 6.4 to 9.4 min. Therefore, after these tests, it was decided to adopt the following instrumental parameters: injection interval = 170; injection volume = 2; stepwise time 1 = 20; stepwise time 2 = 77; stepwise time 3 = 140; stop time = 9900; off time = 800; response time = 1.00 (for all the minor fractions).

The flow is critical for optimal resolution of the Hb F peak: a flow of 1 ml/min is obtained by setting the internal knob to a position between 25 and 30. The operating pressure, under such conditions, is between 55 and 60 kg/cm². By using these parameters the haemoglobins are eluted in the following order (retention times in parentheses, and given in min): Hb A_{1a} and A_{1b} (1.7), A_{1c} (3.1), F (3.9), A₀ (7.5), A₂ (9.9); each run takes approximately 16.5 min.

Columns and reagents used within this evaluation were from Bio-Rad HF and KK batches. All the chromatograms in figures 1–3 have been redrawn from the original paper print-out.

Reference methods

Alternative chromatographic procedures for Hb A₂ have been performed either according to ICSH recommendations [3, 17] or by using disposable microcolumns (Beta-Thal Quick Column, Helena Laboratories, Milan, Italy). The average within-run imprecision for Hb A₂ determinations by these methods (data from two different laboratories) are:

- (1) ICSH method: CV 14.3% (Hb A₂ 2.58%) and 3.7% (Hb A₂ 5.86%).
- (2) Microcolumns: CV 2.8% (Hb A₂ 2.49%) and 1.2% (Hb A₂ 5.22%).

As Hb F reference methods the following were used:

- (a) Alkali denaturation, method 1 [9].
- (b) Alkali denaturation, method 2 [11].
- (c) Radial immunodiffusion, for samples with Hb F lower than 12%.
- (d) Diamat analysis for Hb A_{1c} (see later).

The within-run imprecisions of these methods (as CV) for Hb F concentrations between 0.8 and 2.2% are: 7.3% (method a), 8.0% (method b), 4.3% (method c) and 3.3% (method d).

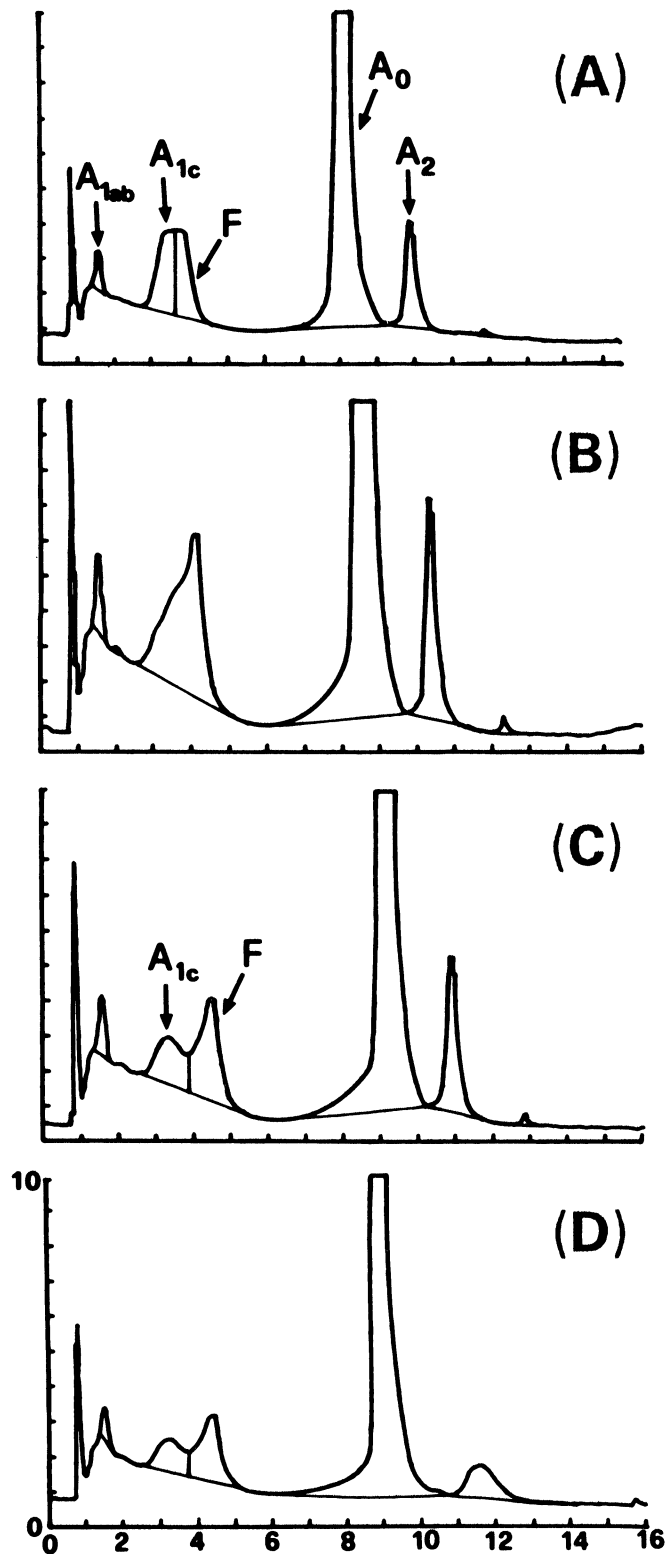


Figure 1. Effect of the elongation of buffers elution time (e.t.) on the resolution between Hb A_{1c} and Hb F. (a): instrumental parameters as recommended by Bio-Rad Laboratories; (b)–(d): tested modifications. (a): Buffer 1 e.t.: 1.0 min; buffer 2 e.t.: 6.4 min. (b): Buffer 1 e.t.: 1.5 min; buffer 2 e.t.: 6.4 min. (c): Buffer 1 e.t.: 2.0 min; buffer 2 e.t.: 6.4 min. (d): Buffer 1 e.t.: 2.0 min; buffer 2 e.t.: 9.4 min. The ordinate axis (absorbance) is automatically set to a fixed percentage of full scale (typically 10%), when Hb A_{1c} is precisely located. The x-axis is the elution time (min). The scales of graphs (a)–(c) are the same as for (d).

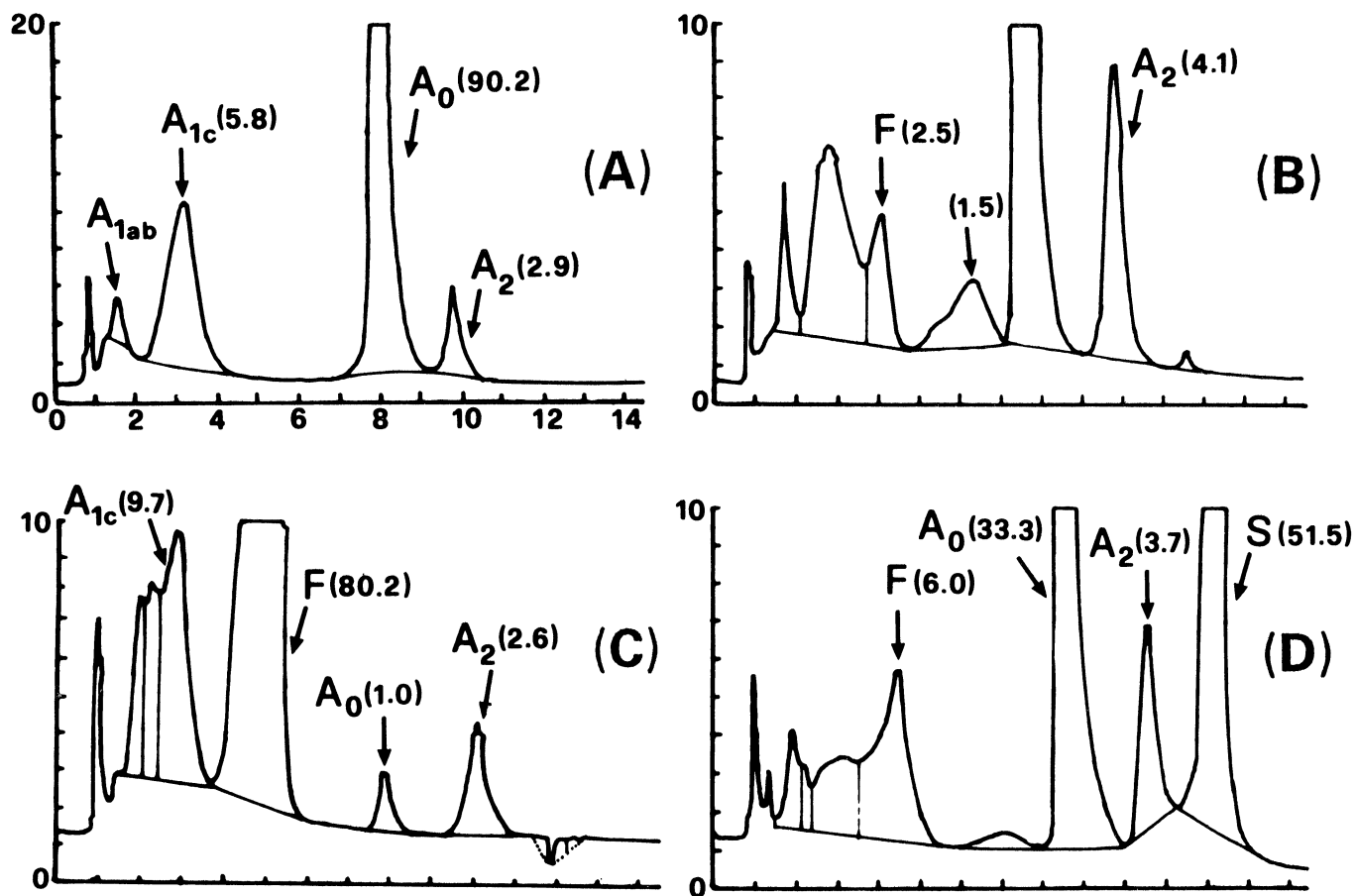


Figure 2. Chromatograms obtained from the analysis of a normal subject (a), a β -thalassaemia carrier (b), a β^0 -homozygous (c) and a double heterozygous for β -thal and Hb S (d). The relative concentrations of the different haemoglobin components are reported in parentheses, in % unit. (b), (c) and (d) x-axis scales as in (a).

As Hb A_{1c} reference method another Diamat instrument was used, which was dedicated to Hb A_{1c} measurement for the diabetic centre (later called the x-method). This instrument is equipped with a different column and buffer elution system; and its analytical characteristics have previously been evaluated [12–14]. Each blood sample was therefore treated independently for each analysis and loaded on the two instruments almost simultaneously (or at least no longer than 12 h between the two determinations).

Statistical analysis

All the statistics have been calculated on an IBM personal computer by using parametric tests. Hb A₂ values in normals and in β -thal carriers were found to be normally distributed, as evaluated by Kolmogorov-Smirnov test [18] and asymmetry and kurtosis coefficients. The determination of the reference limits was performed according to the IFCC recommendations [19].

Results

Chromatographic resolution of minor and abnormal haemoglobins

Some typical chromatograms obtained by the Diamat analyser are reported in figures 2 and 3. As can be seen from figure 2(a), Hb F is normally not resolved if its

concentration is below 1%. When an abnormal peak is isolated, this is reported as an unknown fraction which has to be interpreted from the chromatogram. The small peak between Hb F and Hb A₀ (figures 2[b] and [d]) is usually absent, and, if present, never exceeds 1.5% of total haemoglobin. This can probably be identified as Hb A_{1d}, an adduct of haemoglobin and oxidized glutathione [20].

Surprisingly, in the case of β -thalassaemic homozygous (figure 2[c]) a small percentage of Hb A₀ and an abnormal Hb A_{1c} peak was found. Complete absence of Hb A₀ in this subject has been demonstrated by DNA analysis with synthetic oligonucleotides [21], which showed a mutation at codon β 39 (CAG \rightarrow TAG). This kind of mutation is responsible for more than 95% of all cases of β -thalassaemia in Sardinia. Therefore, the small fraction detected as Hb A₀ is probably an artefact, or some unidentified component.

As regards to Hb A_{1c}, a significant amount of acetylated Hb F is probably responsible for the elevation of the peak; from a comparative study with isoelectric focusing on cord blood samples, it has been shown that Hb F is eluted among Hb A₁ fractions (data not shown here).

Hb H and Hb Bart's (figures 3[a] and [b]) are eluted before the Hb A₁ fractions; this is in agreement with data

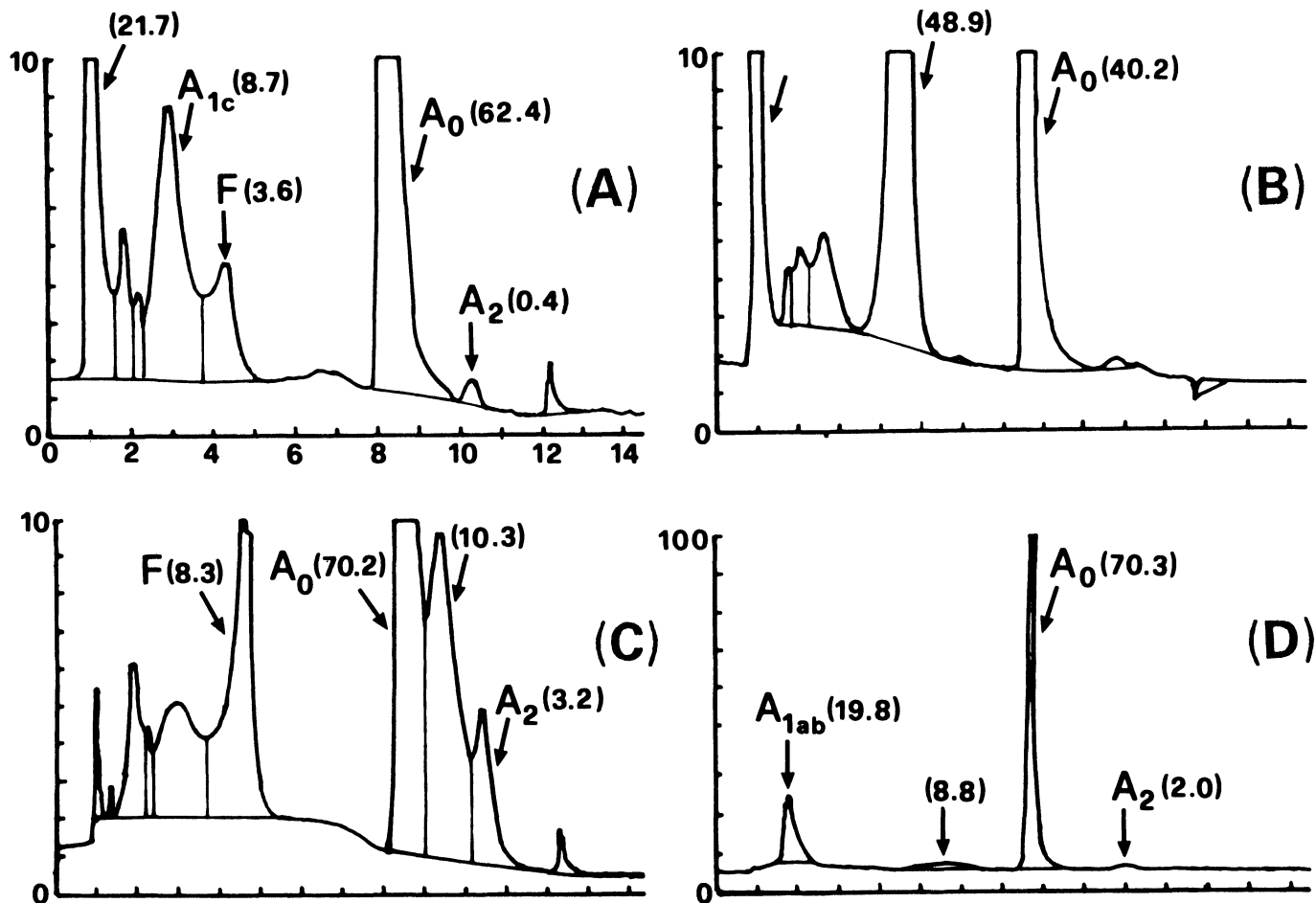


Figure 3. Chromatograms from: a subject suffering from Hb H disease (a), a newborn with Hb Bart's (b), an Hb Lepore (c) and an Hb J Sardegna carrier (d).

reported when using a similar cation-exchange HPLC procedure [20]. Hb Lepore (figure 3[c]) is slightly underestimated with respect to densitometric analysis (10.3% versus 15.0%, respectively).

Figure 3(d) reports on a case of a Hb J Sardegna ($\alpha 50$ [CE8] His \rightarrow Asp) carrier; as frequently happens in such cases, if the Hb A_{1c} is not resolved, the absorbance scale is automatically set to 100%. The haemoglobin variant is eluted among the Hb A₁ fractions in agreement with its 'fast' electrophoretic mobility and is correctly quantitated (about 19%, against the 20.5% obtained by densitometry of the electrophoretic trace).

Accuracy of Hb A_{1c} determination has been tested by analysing several samples, from normal and diabetic subjects, using the two Diamat procedures. The results of such a comparison are reported in figure 4.

Analytical performance

Total imprecision of the method was tested separately for Hb A₂ and Hb F by analysing several samples from normals and β -thal carriers. The results are reported in

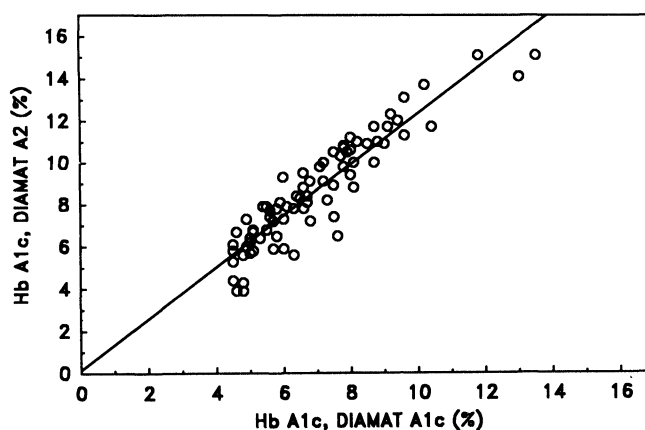


Figure 4. Comparison between the present method (y) and a reference method (x) for Hb A_{1c} quantification: $y = 1.217x + 0.16$; $n = 79$; $r = 0.9235$; $S_{yx} = 0.99$.

table 1. The Hb A₂ determination is highly reproducible (CV never exceeding 4%). Acceptable imprecision has also been found for Hb F; only for concentrations in the range of 1% or lower are the CVs significantly higher.

Linearity was evaluated by measuring in duplicate different haemoglobin solutions prepared by mixing Hb

Table 1. Analytical imprecision.

| | Hb A ₂ | | | Hb F | | |
|--------------------|-------------------|-------------|-----|------|-------------|------|
| | N | CV | | N | CV | |
| | | Mean ± SD | % | | Mean ± SD | % |
| Within-Run | | | | | | |
| Normal | 10 | 2.71 ± 0.07 | 2.6 | 10 | 1.30 ± 0.07 | 5.1 |
| Abnormal-1 | 10 | 5.22 ± 0.15 | 2.9 | 10 | 3.47 ± 0.14 | 4.1 |
| Abnormal-2 | — | — | — | 10 | 7.94 ± 0.20 | 2.5 |
| Between-run | | | | | | |
| Normal | 15 | 2.82 ± 0.10 | 3.6 | 17 | 0.71 ± 0.19 | 27.0 |
| Abnormal | 15 | 4.03 ± 0.16 | 3.9 | 22 | 3.91 ± 0.13 | 3.2 |

A₀ and Hb A₂, isolated by conventional liquid chromatography (Hb A₂ linearity test) or saline washed erythrocytes obtained from a normal subject and cord blood (Hb F linearity test).

The HPLC procedure for these measurements is linear for Hb A₂ concentrations up to 6% ($y = 0.94x + 0.45$; $r = 0.992$; $S_{yx} = 0.22$) and for Hb F values up to 34% ($y = 1.42x - 0.37$; $r = 0.998$; $S_{yx} = 0.88$). However, acceptable Hb F measurement can be taken even at higher Hb F concentrations, up to 60% ($y = 1.08x + 2.44$; $r = 0.990$; $S_{yx} = 3.36$).

Accuracy was tested by making a comparison of the Diamat proposed procedure against several reference methods, as reported in table 2. The correlations between these methods were excellent.

Finally, the procedure has no carry-over and is insensitive to changes in sample total haemoglobin, in the range 4.4–20.4 g/dl (data not reported in detail).

With regards to column life, we found that Hb F can be properly resolved for up to 750–800 runs. Up to 1100 runs of Hb A₂ can be made.

Reference values

Hb A₂ reference values were evaluated for normal adults and β-thal carriers. From the statistical analysis of elementary data the following values were obtained:

- (1) Normals (N = 597) Mean ± SD: 2.64 ± 0.32
95% confidence interval: 2.02 – 3.27%
- (2) β-thal carriers (N = 200) Mean ± SD: 4.91 ± 0.51%
95% confidence interval: 3.92 – 5.90%.

The cut-off limit can be set at Hb A₂ = 3.35%; out of the 797 subjects only three had Hb A₂ values between 3.4 and 3.6%.

Hb F values in normals and in different thalassaemia syndromes carriers were distributed as follows. Hb F concentrations higher than 1.0% were measured in:

- (a) 44 out of 192 normal young subjects (age 1–14 years, 22.9%) and in 41 out of 776 normal adults (5.3%).
- (b) 38 out of 65 β-thal young carriers (age 1–14 years, 58.5%) and in 81 out of 237 β-thal adults (34.2%).

Hb F concentrations in three non-deletion δβ-thalassaemia carriers, Sardinian type [22] were: 14.1 ± 1.7% (mean ± SD).

Discussion

The authors first experiences with the Diamat system applied to the determination of haemoglobin species in blood are reported. It is clear that this apparatus can improve routine haematological management in the clinical laboratory. The system offers the following advantages:

- (1) Hb A₂ is measured accurately and reproducibly. This is relevant because Hb A₂ accounts for only a small amount of total haemoglobin (Hb A₂ concentrations higher than 6% are rarely found) and because the separation limit between normals and β-thalassaemics is very narrow. A similar reproducibility is rarely obtained with manual chromatographic methods.

The reference intervals found for normals and β-thalassaemia carriers are in agreement with those already reported [3].

Table 2. Relevant linear regression parameters concerning the comparison among the Diamat method (y) and different techniques for Hb A₂ and Hb F determination (x-methods).

| | x-method | N | Slope ± SD | Int. ± SD | S _{yx} | Range, % | r |
|-------------------|--------------------------|-----|---------------|--------------|-----------------|------------------------|--------|
| Hb A ₂ | Ion-exchange | 70 | 0.883 ± 0.030 | 0.33 ± 0.12 | 0.30 | 2.0–6.0 ^a | 0.9639 |
| | Ion-exchange (Helena) | 200 | 0.942 ± 0.015 | 0.36 ± 0.06 | 0.28 | 1.6–5.9 ^a | 0.9755 |
| Hb F | Alkali den. ^c | 30 | 1.078 ± 0.011 | −0.60 ± 0.20 | 0.98 | 1.3–55.0 ^b | 0.9990 |
| | Alkali den. ^d | 49 | 1.016 ± 0.005 | −0.12 ± 0.23 | 0.83 | 12.0–80.0 ^b | 0.9995 |
| | Radial imm. ^d | 31 | 1.040 ± 0.031 | 0.17 ± 0.17 | 0.52 | 0.5–12.0 ^b | 0.9877 |
| | Diamat-A _{1c} | 31 | 1.030 ± 0.013 | 0.20 ± 0.30 | 1.39 | 1.1–65.0 ^b | 0.9980 |

a: Minimum and maximum Hb A₂ concentrations determined by x-method.
 b: Minimum and maximum Hb F concentrations determined by x-method.
 c: According to ref. [11].
 d: According to ref. [9].

- (2) Simultaneous Hb F determination eliminates the need for separate analysis; this kind of measurement is, for most Hb F assay protocols, time-consuming, poorly reproducible and seldom linear to high concentrations.
- (3) Chromatographic analysis is able to discriminate between several haemoglobin variants; their quantifications is, in the majority of cases, in agreement with the electrophoretic pattern.
- (4) Sampler capacity (48 tubes) and the system's automation allow up to 90 samples per day to be analysed; no special skills are required to operate the instrument.

A cation exchange HPLC analysis recently proposed by Bisse and Wieland [20] seems to offer similar advantages. The system optimized by these authors is probably superior, in terms of resolution, but compared to the Diamat procedure, has the following drawbacks:

- (a) The analysis is more time-consuming (each run takes more than 60 min, while the Diamat run is only one-third as long).
- (b) No information is available on accuracy in Hb A₂ measurement.
- (c) Hb A_{1c} accuracy has been evaluated on a small number of subjects ($N = 25$), with only three of these subjects out of normal range.

No other fast high resolution HPLC procedure for the separation of Hb F, Hb A₀, Hb A₂ and other Hb variants seems to have been presented.

The Diamat system has some minor problems. For example Hb A_{1c} quantification seems to be overestimated with respect to a reference method of proved accuracy [12]. A possible explanation for this could be the influence of labile aldimine forms which, if not removed during the haemolysis step, can co-migrate with the Hb A_{1c} component (stable ketoamine form). The importance of removing these labile forms has been stressed by several investigators [23–25]. It has also been demonstrated that the Diamat A_{1c} analysis (the x-method in figure 4) is specific for the stable forms [25]. In fact, a significative difference has been found by analysing the haemolysing agent used for Hb A_{1c} determination (x-method) and the one used with the test method (y-method in figure 4); the two agents differ in pH (5.95 versus 7.20, respectively) and in conductivity (246 versus 132 μ S, respectively). In order to confirm that the haemolysing agent has a powerful effect on Hb A_{1c} concentration, Hb A_{1c} was measured using the proposed method, in a sample treated with the two haemolysing agents separately. The following values were found: 5.3% (x-method haemolysing solution) and 7.3% (y-method haemolysing solution). In conclusion, the Hb A_{1c} measurement on Diamat equipped with reagents and column for Hb A₂ and Hb F is probably inaccurate, because the labile aldimine form is not removed during the haemolysis step.

A second limitation of the technique concerns peak identification. This is easily done for normal samples,

but, when dealing with abnormal samples (for example those reported in figures 2 and 3) peak identification should be performed by an experienced haematologist. It is also evident that, if a pathological result is obtained, further analyses, such as DNA analysis and globin chain synthesis, have to be made in order to obtain a final diagnosis.

Apart from these limitations, this kind of HPLC analysis is useful for diagnosis of haemoglobin-related disorders. Improvements, such as a further reduction of analysis times for a correct estimation of Hb A_{1c}, would be useful.

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