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Assessment of an Automatic Liquid Chromatograph for Foetal and Abnormal Haemoglobins

By *S. Brenna, L. Prencipe, S. Granata* and *N. Montalbetti*

Laboratory of Clinical Biochemistry and Hematology Ca' Granda — Niguarda Hospital, Milan, Italy

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Summary: The DIAMAT TM (Bio-Rad) analyser is a microprocessor-operated HPLC system using a silica-based weak cation exchange column with three phosphate buffers of increasing ionic strength as the step gradient mobile phase. A dual wavelength detector measures absorbance at 415 and 690 nm; each sample is completely processed in 8 minutes. The instrument effectively separates and quantifies HbF in a discrete peak. We have verified that the HbF assay is linear up to about 65% values.

We calculated within-run imprecision for n = 20 in 3 different haemolysed blood samples; the results are shown below as percent of total haemoglobin:

A)	Mean = 1.06	SD = 0.048	CV% = 4.5
B)	Mean = 1.90	SD = 0.041	CV% = 2.1
C)	Mean = 8.93	SD = 0.047	CV% = 0.5

Between-run imprecision for similar samples (n = 18) was:

D)	Mean = 3.74	SD = 0.20	CV% = 5.5
E)	Mean = 9.94	SD = 0.15	CV% = 1.5

Accuracy was assessed in different series by correlating Hb values (y) with those obtained by the alkali denaturation test (x). The regression line equation was $y = 1.03\,x - 0.33$ ($r = 0.999$, $n = 62$).

The DIAMAT TM instrument also reveals the presence of any HbS and calculates its peak area correctly, as we found by electrophoretic reassay in heterozygous subjects. We also noted the presence of other abnormal haemoglobins characterized by specific retention times.

Introduction

In normal adult blood, foetal haemoglobin (HbF) accounts for not more than 2% of total haemoglobin. HbF up to 20% of total can be found in heterozygous cases of β-thalassaemia, hereditary persistence of foetal haemoglobin (HPFH), and in double heterozygosis with β-thalassaemia plus one haemoglobin variant such as HbC, HbS or HbE. Finally, HbF concentrations exceeding 70% of total Hb occur in homozygous β-thalassaemia, δ-β-thalassaemia, and hereditary persistence of foetal haemoglobin.

One widely used method for assaying HbF is based on its resistance to alkali denaturation (1); this method, however, is poorly sensitive to high HbF concentrations.

Two methods used routinely are haemoglobin electrophoresis on cellulose acetate at an alkaline pH (2) and on agar citrate at an acidic pH (3), each being suitable for separating different haemoglobins.

Isoelectric focusing affords excellent resolution (4); but the method is remarkably complex and its use in the clinical chemistry laboratory is restricted for the time being to research work.

Other methods for assaying HbF are radial immunodiffusion (5), cation-exchange micro-chromatography for eluting "fast" haemoglobins (6), and some variants of ion-exchange high-performance liquid chromatography (HPLC) (7, 8).

The DIAMAT TM apparatus is a high performance liquid chromatography analyser originally intended for the automatic assay of glycated haemoglobins. The same apparatus, however, also quantifies HbF in a separate peak (fig. 1), as well as HbS, if any, and other abnormal peaks.

The possibility of complete automation of these assays, offered by the DIAMAT TM instrument, prompted us to test it for accuracy and reliability in the screening of abnormal haemoglobins, primarily HbF. The more laborious methods of electrophoresis and alkali resistance were used for confirmation.

TIME 86-07-15 16:47

SAMPLE NO. 011

NAME	%	TIME	AREA
A1AB	3.2	2.5	189.71
F	16.8	3.4	1001.79
A1C	3.1	4.4	169.57
A0	76.9	6.1	4574.13

HBA1C 3.1 % HBA1 6.2 %

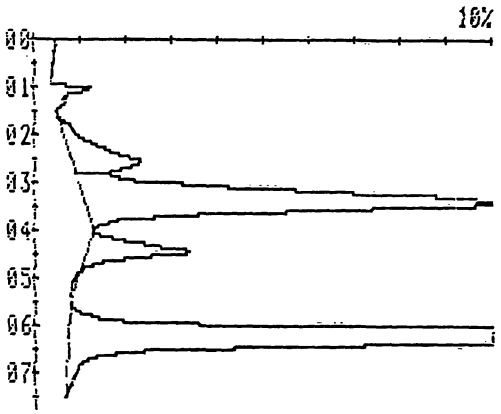

Fig. 1. Printout of sample with high HbF.

Materials and Methods

High performance liquid chromatography analyser

The Bio-Rad DIAMAT TM instrument is an automatic analyser utilizing the principles of ion-exchange high-performance liquid chromatography for assaying glycated haemoglobin. The apparatus features a single piston pump with a step-gradient valve system that allows three phosphate buffers of increasing ionic strength and different pH to run through the analytical column in a timed sequence.

First solution:	phosphate buffer,	90 mmol/l	pH = 5.9
Second solution:	phosphate buffer,	140 mmol/l	pH = 5.8
Third solution,	phosphate buffer,	270 mmol/l	pH = 5.7

The column (4 mm i.d. × 15 cm) is packed with a weak cation-exchange resin in bead form; the support is silica with a carboxymethyl functional group. Buffers are eluted in the following order:

buffer 1: 1.7 minutes,
buffer 2: 2.0 minutes,
buffer 3: 2.1 minutes and
buffer 1: 2.2 minutes.

Thus, each sample is completely processed in 8 minutes. Haemolysed blood samples are stored in the refrigerated autosampler chamber until a 5 µl portion is automatically injected into the column thermostated at 23 °C.

Readings are made at 415 and 690 nm, to eliminate problems of turbidity or light source instability.

All operations are controlled by a microprocessor. The printout is a summary of HbA_1c and HbA_1 (a + b + c) values as well as of the relative (area) percentages of all resolved haemoglobins.

Alkali denaturation

We used the method of *Singer* et al. (11).

Alkaline-pH haemoglobin electrophoresis

For this we used the completely automatic OLYMPUS AES 2000 apparatus. Electrophoresis was conducted on SARTORIUS cellulose acetate strips at 4 mA for 35 minutes in TRIS-EDTA-glycine buffer at pH 9.2 with semi-micro seeding, and strips were stained with Ponceau Red.

Sample treatment

For comparison and reproducibility tests in the DIAMAT TM series we used fresh venous blood samples with EDTA added as anticoagulant. Before analysis, the blood was haemolysed and diluted 1:200 with a solution of polyoxyethylene ether in borate buffer, volume fraction 0.001.

In such haemolysates, even if stored at −20 °C, HbF is not very stable from day to day; thus for assessing between-run precision we used two blood samples of high and low HbF content stored in the refrigerator.

For haemoglobin electrophoresis, suitable portions of whole blood were washed repeatedly with isotonic saline solution. We then treated 1 volume of red blood cells with 1 volume of water and 1/2 volume of toluene.

Results

Linearity

To verify the linearity of HbF assays we mixed a sample of neonatal blood (HbF = 85%) in 20 different portions with adult blood of equal haemoglobin content and no appreciable HbF content, thus making 20 serial dilutions of HbF from 0 to 85%.

From the first 4 points (0 to 12% HbF) we calculated the linear regression for the HbF values expected from dilution and those actually produced by the DIAMAT TM instrument.

The remaining points were never more than 5% away from the theoretical line, except for HbF contents exceeding 65%.

Precision

Within-run precision was assessed in 20 replicates of 3 blood samples with different HbF contents.

HbF readings are expressed as percentage of total Hb:
Sample A: mean = 1.06, SD = 0.048, CV% = 4.5
Sample B: mean = 1.90, SD = 0.041, CV% = 2.1
Sample C: mean = 8.93, SD = 0.047, CV% = 0.5

The between-run precision test (18 replicates of 2 pathological samples) gave:
Sample D: mean = 3.74, SD = 0.20, CV% = 5.5
Sample E: mean = 9.94, SD = 0.15, CV% = 1.5

Method comparison

Accuracy was assessed in different series by correlating HbF values (y) with those obtained with the alkali-denaturation method (x) in the range of 0 to 70% HbF. The linear regression equation was:

$$y = 1.03\,x - 0.33;\ r = 0.999;\ N = 62$$

Carry-over

We investigated sample related carry-over in the DIAMAT TM instrument by performing 5 determinations of a high-HbF blood sample (70%) followed by 5 determinations of a low-HbF sample (0.5%). We repeated the experiment 10 times, and then calculated the mean value of the ratio:

$$K = \frac{(l_1 - l_5)}{(h_5 - l_5)}$$

The resulting "carry-over constant" of 1.4×10^{-3} indicates the absence of sample-to-sample carry-over or incomplete column elution.

Table 1 shows the results of HbS assays in some subjects with the HbS trait.

Tab. 1. HbS percentage in 4 subjects with HbS-trait

Patient	DIAMAT TM	Electrophoresis
A	46 %	47 %
B	45.9%	44.3%
C	41.8%	42 %
D	73 %	72 %

Discussion

Among the various programs offered by the DIAMAT TM instrument, the basic routine program affords the elution and assay of glycated haemoglobin fractions and HbF in only 8 minutes.

Chromatography proved efficient and precise, especially for pathologic HbF values.

The HbF assay is linear up to 65% values; comparison with the alkali-resistance method revealed good correlation, even at high HbF values.

Thus the DIAMAT TM apparatus affords ready detection of most cases of β-thalassaemia and HbF persistence without recourse to the more laborious and time-consuming method based on alkali resistance.

The same DIAMAT TM program also reveals HbS, which is eluted next to HbA (fig. 2). In such cases the instrument does not indicate the percentage of

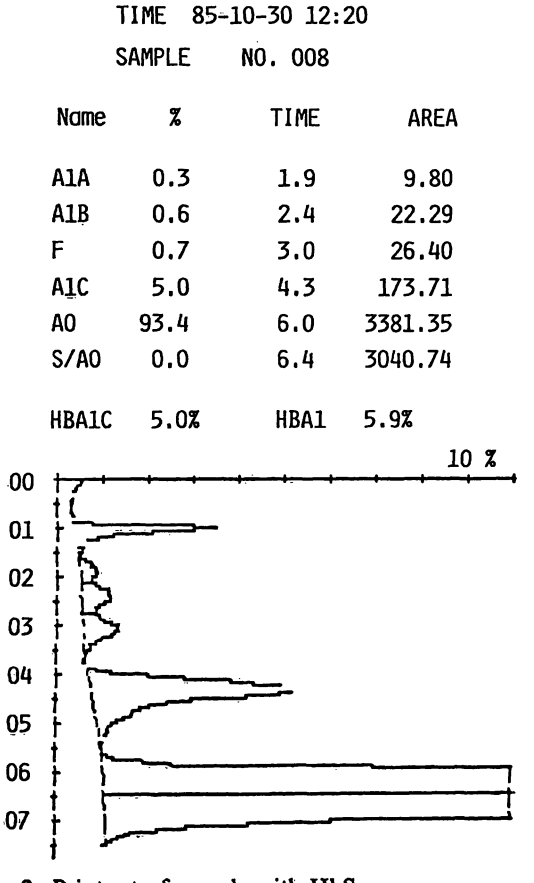

Fig. 2. Printout of sample with HbS.

HbS directly but only its peak area, from which the percentage relative to total haemoglobin is readily calculated. The HbS area calculated by the integrator is in good aggreement with the percentage found by electrophoresis.

As for the possible detection and assay of other haemoglobin subfractions, we are currently testing some software modifications and different elution buffers; preliminary results appear promising for HbC and HbA_2.

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Dr. Sergio Brenna
via Campo dei Fiori, 32
I-20026 Novate Milanese