Technical method

A simple micromethod for the measurement of fetal haemoglobin

B. E. SERJEANT, J. M. CLARKE, P. DESAI, G. R. SERJEANT,
*MRC Epidemiology Unit, University of the West
Indies, Kingston, Jamaica*

Most modern assay methods of fetal haemoglobin (Hb F) are based on its relative resistance to denaturation by strong alkali, a property first noted by Korber in 1866. When haemolysates containing Hb A and Hb F are exposed to alkali, the denaturation reaction demonstrates two components, the first rapid reaction related predominantly to the denaturation Hb A and the second exponential component presumed to be related to the slower denaturation of Hb F.

Determination of HbF has depended on either measurement of the denaturation rate under standardized conditions (Brinkman and Jonxis, 1935; Singer *et al*, 1951a; Beaven *et al*, 1960a) or measurement of the unaltered haemoglobin remaining after denaturation has been allowed to continue for a precisely timed interval (Singer *et al*, 1951b). The latter method is simpler although there is evidence that it tends to underestimate high values of Hb F (Jonxis and Huisman, 1956). Additional modifications of this method that have since been proposed include the use of haemolysates in the cyanmethaemoglobin form which avoids errors caused by methaemoglobin and carbonmonoxyhaemoglobin derivatives (Betke *et al*, 1959) and variation in the optimum wavelength at which the samples are measured (Jonxis and Visser, 1956; Kristoffersen, 1961; Pembrey *et al*, 1972). Automated assay systems based on this method have also been developed (Cabannes and Schmidt-Beurrier, 1965; Brook *et al*, 1974). The most common currently used tests are those of Singer *et al* (1951b) and Betke *et al* (1959) giving values for the upper limit of normal of 2% and 1% respectively. Both methods appear to be highly reliable for low levels of Hb F but may underestimate high levels.

In a current cohort study of sickle cell disease following diagnosis at birth, the need has arisen for a method of Hb F estimation capable of comparing the fall of Hb F levels in homozygous sickle cell disease (SS) with that in normal controls with the AA genotype. The method is required to be rea-

sonably accurate over a wide range of Hb F levels and to be capable of being performed on small blood samples obtained by heel prick. A micro-modification of the method of Singer *et al* (1951b) has been developed and appears to give satisfactory results.

Method

REAGENTS

Haemolysing reagent-Stock solution (40 g/dl tetrasodium ethylenediaminetetra acetate) diluted 0.3 ml in 100 ml distilled water for working solution (Schneider, 1973). Potassium cyanide (KCN 2 g/dl). Half-saturated ammonium sulphate (400 ml saturated ammonium sulphate; 400 ml distilled water; 2 ml 10M HCl) stored at 4°C in a plastic bottle. 0.08 M sodium hydroxide stored at 4°C in a plastic bottle.

PREPARATION OF HAEMOLYSATE

Following heel prick, a heparinized microhaematocrit tube is three-quarters filled with capillary blood (approximately 40 μ l), flame sealed, and spun in a microhaematocrit centrifuge for 5 minutes. The column of packed cells is removed by cutting the tube above the sealed bottom and again just below the buffy layer. This glass segment is placed in a narrow bore test tube (75 $\times$ 10 mm) containing 3-4 drops of haemolysing reagent which is then centrifuged for 2 minutes. Brisk mixing removes any remaining cells from the capillary tube which can then be removed. The haemolysates in each batch are matched visually by the addition of further haemolysing reagent resulting in a volume of 0.3-0.4 ml (sufficient for duplicate tests) and a concentration of approximately 1-2 g/dl. One drop of KCN (2 g/dl) is added, and, after standing for 5 minutes, the haemolysate is centrifuged and the clear supernatant is removed to a clean tube.

TEST PROCEDURE

The test is performed in a water bath adjusted to 20°C. One ml of 0.08 M sodium hydroxide is placed in one test tube and 2 ml of half-saturated ammonium sulphate is placed in a second test tube, both tubes being temperature equilibrated in the water bath for 10 minutes. Haemolysate, 0.05 ml, is added to the tube containing 1 ml of 0.08 M sodium hydroxide, and a stop watch is started at the same time. The mixture is rapidly rinsed up and down the pipette five times to ensure that all the haemolysate is properly mixed. After exactly 60 seconds, the contents of both tubes are mixed together by pouring from one tube to the other five times. After standing for 5 minutes, the mixture is filtered through a 7 cm

diameter Whatmann No. 42 filter paper in a 5 cm diameter funnel. An undenatured 'total' sample is prepared by adding 0.02 ml of haemolysate to 3 ml distilled water, and both samples are read in a spectrophotometer at 540 nm.

CALCULATION

The dilution of the test sample is 1/61 (0.05 ml in 3 ml) and of the 'total' sample 1/151 (0.02 ml in 3 ml). The ratio of the dilutions is therefore $61/151 \times 100$ or 40.4. The percentage of alkali resistant haemoglobin may therefore be calculated from the formula:

$$\frac{\text{OD of test sample}}{\text{OD of total sample}} \times 40.4$$

ASSESSMENT OF TECHNIQUE

Accuracy

The method was tested by comparing results with the known concentrations of mixtures of pure Hb A and Hb F prepared by DEAE-Sephadex chromatography. Duplicate estimations were performed on haemolysate concentrations of 2g, 1g, and 0.5 g/dl (fig 1). Measurements by the micromethod rose in a near linear pattern with rising levels of Hb F although the method increasingly underestimated Hb F levels above 10%, a feature characteristic of other alkali denaturation techniques.

REPRODUCIBILITY

The results of 10 estimations on each of three haemolysates (concentration 1-2 g/dl) with different levels of Hb F are shown in table I. Reproducibility was excellent at the two higher levels of Hb F but was worse at the 0.8% level because of the high percentage variability of small errors at such low levels. With a haemolysate concentration of 0.5 g/dl, agreement between duplication estimations was reduced (fig 1).

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Series	No.	Mean	SD	SE of Mean	95% Confidence Limits of Mean	Coefficient of Variation %
1	10	0.78	0.25	0.08	0.62-0.94	32%
2	10	7.53	0.42	0.13	7.27-7.79	6%
3	10	13.02	0.65	0.21	12.62-13.42	5%

Table I Reproducibility of micromethod at three different levels of Hb F and haemolysate concentration of 1-2g/dl

COMPARISON WITH METHOD OF BETKE *et al*

The results of the micromethod performed on rapid haemolysates and of the technique of Betke *et al* (1959) on standard haemolysates are compared in figure 2. At higher levels there was relatively close agreement between estimations by the two methods but a discrepancy was obvious at low levels. The upper end of the normal range in the Betke method (1%) corresponds to a level of about 4% in the

Fig 1 Relationship between Hb F measured by micromethod and real levels obtained by mixing haemolysates containing pure Hb A and pure Hb F. Duplicate estimates performed on haemolysate concentrations at 2% (closed circles), 1% (open circles), and 0.5% (crosses).

Fig 2 Relationship between % Hb F measured by micromethod on rapid haemolysates and that measured by the technique of Betke *et al* (1959) on standard haemolysates in 56 samples of venous blood from either SS patients (closed circles) or AA controls (open circles).

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Fig 3 Relationship between % Hb F measured by the micromethod and that measured by the technique of Betke *et al* (1959) when both techniques were performed on standard haemolysates.

micromethod. This difference appeared to be largely due to impurities in the rapid haemolysate since a close correlation occurred when both techniques were performed on standard haemolysates (fig 3).

Haemolysate Concentration

The strength of haemolysate obtainable in practice is dependent on the amount of blood available and the volume required and rarely exceeded 1-2 g/dl. The test was most accurate at this haemolysate concentration since at 0.5 g/dl agreement between duplicates and the linear relationship with rising Hb F levels were poor (fig 1). With pure Hb A, the lowest values for alkali-resistant haemoglobin were obtained with the stronger haemolysates.

Normal Values

The cord blood levels and the pattern of decline of alkali-resistant haemoglobin in the AA genotype are summarized in table II and correspond closely to values observed in previous studies (Brinkman and

Jonxis, 1935; Chernoff and Singer, 1952; Beaven *et al*, 1960b; Jonxis, 1961; Schneider, 1973). Estimations with this technique on 20 normal adults gave a mean level of 2.6%. These results indicate an increase in the upper end of the normal range with the micromethod to about 4%, a figure compatible with comparisons with the Betke technique (fig 2).

Discussion

The need for a single method of fetal haemoglobin estimation from the high levels present in the cord blood to the low levels which may be present at 1 year of age inevitably leads to some loss of accuracy. More precise data would be obtained by selection of different methods specifically suited to high or low levels of Hb F but conversion from one method to another with falling Hb F levels would confuse interpretation of the data. The Singer method was chosen for miniaturization rather than the method of Betke *et al* because the majority of expected values were in medium and high ranges of Hb F at which the Singer method appears more reliable. However, estimations of low levels of Hb F are less accurate with the Singer technique. Levels of up to 4% were found in normal adults with the present micromethod, reflecting in part the higher levels obtained in adults with the Singer method (up to 2%) and, in part, the increased values occurring with haemolysate concentrations below 5 g/dl and assumed to result from incomplete denaturation of Hb A in dilute solutions (Pembrey *et al*, 1972). Higher readings are also inevitable when the optical density of the test sample is expressed in relation to that of a weak 'total' sample. Haemolysate concentrations greater than 1-2 g/dl were not possible if sufficient volumes for duplicate estimations were to be made from the small amounts of blood available in the present study. However, the performance of the test appeared satisfactory at this level although error increased with weaker haemolysates.

Another limitation resulting from the small blood sample was the inability to make standard haemolysates. When tests were performed on the whole blood, falsely high values were obtained. Washing the red cells of a small sample was not practicable but spinning in a microhaematocrit tube served to remove much of the plasma, and estimates on the packed red cells gave consistently lower values than when the test was performed on whole blood. When the micromethod and the Betke technique were both performed on standard haemolysates, there was close agreement between the estimates and it is likely that at least part of the discrepancy noted in fig 3 results from persisting impurities from plasma and cell membranes in the rapid haemolysates. The

Age (mth)	No.	Mean (%)	SD	SE of Mean
Cord blood	75	68.00	8.01	0.92
1	34	61.51	13.41	2.30
2	33	43.87	12.02	2.09
3	17	18.56	6.26	1.52
4	25	11.39	4.12	0.82
6	49	9.10	6.08	0.87
8	23	7.62	2.87	0.60
10	16	6.23	4.21	1.05
12	38	5.34	3.48	0.56

Table II Hb F levels in cord bloods and at ages up to 1 year

haemolysing reagent of Schneider (1973) proved a satisfactory method of making haemolysates and does not appear to interfere with the estimation of the Hb F *per se* (Schmidt *et al*, in press).

The underestimation of high Hb F levels apparent from fig 1 conflicts with the observation that Hb F levels measured in the cord blood by the micro-method agree with those reported elsewhere. It is possible in this case that the technical underestimation is offset by falsely high values from impurities persisting in the haemolysate and from the low haemolysate concentration.

The test proved simple and rapid. It was possible to complete tests and reading in the spectrophotometer at a rate of 15-20 samples per hour.

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