The estimation of haemoglobin A₂: Is visual assessment reliable?

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SYNOPSIS Haemoglobin A₂ in 11 normal and 42 B thalassaemia minor subjects was estimated by simple visual assessment of the electrophoretogram. The results obtained give rise to doubts as to the reliability of this technique when used to estimate haemoglobin A₂ under normal laboratory conditions.

Investigation of hypochromic anaemia may involve a haematology laboratory in the examination of blood samples to confirm or exclude the diagnosis of B thalassaemia minor. In addition, especially in countries where B thalassaemia is endemic, the diagnosis of the carrier state is increasingly requested because of programmes designed to eliminate B thalassaemia major by genetic counselling.

The great majority of B thalassaemia minor subjects have a raised haemoglobin A₂ and this is detected in many laboratories by the simple visual inspection of the haemoglobin A₂ band on the electrophoretogram. It is widely recognized that such an assessment of haemoglobin A₂ is lacking in scientific accuracy. The purpose of this paper is to report whether the results obtained from such a convenient technique are nevertheless of sufficient quality for clinical diagnostic purposes.

Haemoglobin A₂ may be more accurately quantitated if it is individually eluted from the major haemoglobin A band after either an electrophoretic or chromatographic separation. The haemoglobin A₂ levels of all specimens in this survey were measured using both cellulose acetate electrophoresis and DEAE Sephadex column chromatography. The reproducibility of these two relatively time-consuming techniques was subsequently compared.

Materials and Methods

Fresh anticoagulated (K₂EDTA) blood samples from 11 normal controls and 46 parents or siblings of Cypriot patients with B thalassaemia major were refrigerated and transported to London within 36 hours of venepuncture. Freshly prepared haemoglobin solutions (Lehmann and Ager, 1960) were

adjusted to a concentration of 10 g/100 ml. Four samples from siblings of patients with B thalassaemia major were found after both cellulose acetate electrophoresis and chromatography consistently to have a haemoglobin A₂ level within the normal range. These were withdrawn from the series leaving 42 'abnormal' blood samples.

A control 'high normal' standard (Hb A₂ = 3.2% by repeated cellulose acetate elution) was included in each paper and starch gel electrophoretogram. After electrophoresis, the haemoglobin A₂ was visually assessed by three independent observers.

VERTICAL PAPER ELECTROPHORESIS

Tris buffer pH 9.1 was prepared (Cradock-Watson, Fenton, and Lehmann, 1959) and diluted to half the recommended strength with distilled water. Then 0.01 ml of sample was applied as a spot to Whatman 3 MM chromatography paper which had been dipped in buffer and lightly blotted. The wet unstained strips were assessed after overnight electrophoresis.

HORIZONTAL STARCH GEL ELECTROPHORESIS

The application of haemolysate to each starch gel was standardized and a discontinuous tris-borate buffer system pH 8.6 (Huehns, 1968) was employed. The haemoglobin A₂ concentration of the haemolysate was assessed in the unstained gel after slicing.

CELLULOSE ACETATE ELECTROPHORESIS

A discontinuous tris/barbiturate buffer system was used (Graham and Grunbaum, 1963), 0.02 ml of haemolysate being applied as a streak to each of three 20 × 5 cm strips. The separated bands of haemoglobin A and A₂ from the three strips were eluted into Drabkin's solution, the optical density

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being compared at 413 nm. All estimations were performed in duplicate the mean results being recorded.

COLUMN CHROMATOGRAPHY

The method employed was based on that of Huisman and Dozy (1965). Columns, 30×1 cm, were used with DEAE Sephadex A50 (3.5 ± 0.5 m-equiv/g, size 40-120 μ l) equilibrated overnight in 0.05 M Tris buffer pH 8.2 flowing at 20 ml/hr. Between 0.5 and 0.6 ml of the haemolysate (dialysed overnight against pH 8.2 buffer) was applied to the top of the column, followed by more pH 8.2 buffer. Haemoglobins A₂ and A were then eluted consecutively using the pH 8.0 buffer for three hours followed by the pH 6.5 buffer. Aliquots were filtered and compared photometrically at 413 nm. All estimations were performed in duplicate, the mean result being recorded.

Results

VISUAL ASSESSMENT OF PAPER AND STARCH GEL ELECTROPHORETOGRAMS

The haemoglobin A₂ fractions from 11 normal and 42 abnormal subjects were compared visually by three experienced observers with the 'high normal' reference samples (3.2%) included on each electrophoretogram. Observers were asked to record the haemoglobin A₂ as raised, doubtful, or normal (table I). Using paper, the normal samples were designated as 'raised' on eight, 'doubtful' on 20, and 'normal' five times (total of 3×11 observations). Samples from the abnormal group were designated as 'raised' on 119, and 'doubtful' on seven occasions (3×42 observations). Using starch gel, the normal samples were classified as 'raised' on one, 'doubtful' on nine, and 'normal' on 23 occasions (3×11 observations); and the abnormal samples as 'raised' on 97, 'doubtful' on 25, and 'normal' on four occasions (3×42 observations).

CELLULOSE ACETATE ELUTION

The average haemoglobin A₂ values of the 11 normal and 42 abnormal samples were 2.4% and 4.5% respectively. All haemoglobin A₂ levels from the

normal group were less than 3.2%, and from the abnormal group, greater than 3.6%. Six replicate determinations of the same sample gave a coefficient of variation of $\pm 3.6\%$.

COLUMN CHROMATOGRAPHY

The average haemoglobin A₂ values for the 11 normal and 42 abnormal samples were 2.6% and 5.0% respectively. All haemoglobin A₂ levels from the normal group were less than 3.3%, and only one of the abnormal samples was less than 4.0%. This gave a low value of 3.5%, though by cellulose acetate elution a value of 4.5% was obtained for this sample. Six replicate determinations performed on a single sample gave a coefficient of variation of $\pm 2.2\%$.

Discussion

Table I demonstrated that in 159 visual estimations of the haemoglobin A₂ level made after paper electrophoresis, eight normal samples were classified as raised, there being no misclassification of abnormal haemoglobin A₂ samples as normal. A similar number of observations made after starch-gel electrophoresis gave one misclassification of a normal sample as raised, and in four cases abnormal samples were reported as normal. It is of interest that the misclassifications after paper electrophoresis were spread evenly between the three observers whereas all the misclassifications after starch gel electrophoresis occurred with observer 3. It was this observer who was most prepared to commit himself and therefore least inclined to report results as doubtful. This suggests that a larger number of serious errors would have occurred in this survey if the choice had been limited to normal or raised.

It should also be remembered that the ideal situation created for this survey seldom exists in clinical practice; thus all applications were carefully standardized with respect to quantity and haemoglobin concentration, and a 'high normal' haemoglobin A₂ reference (stored in liquid nitrogen) was included on each electrophoretogram. The relative instability of haemoglobin A₂, when kept as a haemolysate in the ordinary deep freeze, may make

Haemoglobin A ₂ Level Reported after Visual Assessment	Observer 1				Observer 2				Observer 3			
	Paper		Starch Gel		Paper		Starch Gel		Paper		Starch Gel	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
'Raised'	2	40	—	33	3	37	—	33	3	42	1	31
'Doubtful'	8	2	4	9	8	5	5	9	4	—	—	7
'Normal'	1	—	7	—	—	—	6	—	4	—	10	4

Table 1 The visual assessment of haemoglobin A₂ on paper and starch gel electrophoretograms by three observers

these conditions difficult to achieve. Indeed, in laboratory practice the test sample may well be compared with an unquantitated 'normal' blood sample that has been selected from the day's work batch because of the Anglo-Saxon name of the donor.

A reliable technique for the accurate quantitation of haemoglobin A₂ should not only give reproducible results, but also emphasize the 'discriminatory gap' between the haemoglobin A₂ levels found in the normal and B thalassaemia minor ranges.

The results obtained in this survey indicate that elution after electrophoresis on cellulose acetate and column chromatography fulfil both these criteria. The expertise in electrophoresis already possessed by the staff of a routine clinical laboratory suggests that if either of these two techniques is to be established, estimation of haemoglobin A₂ after cellulose acetate electrophoresis is likely to be preferred.

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