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 electrophorogram. 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 electrophorogram. 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 (K₃EDTA) 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 These were withdrawn from the series leaving 42 ‘abnormal’ blood samples.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 electrophorogram. 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 x 5 cm strips. The separated bands of haemoglobin A and A₂ from the three strips were eluted into Drabkin’s solution, the optical density
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 μl) 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. Haemoglobin 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 ELECTROPHORETOMGRAMS**
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 1). 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 ±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 lower 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 ±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 these 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

<table>
<thead>
<tr>
<th>Haemoglobin A2 Level Reported after Visual Assessment</th>
<th>Observer 1</th>
<th>Observer 2</th>
<th>Observer 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paper</td>
<td>Starch Gel</td>
<td>Paper</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Abnormal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Abnormal</td>
<td>Normal</td>
</tr>
<tr>
<td>'Raised'</td>
<td>2</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td>'Doubtful'</td>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>'Normal'</td>
<td>1</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 1 The visual assessment of haemoglobin A2 on paper and starch gel electrophoretograms by three observers.
The estimation of haemoglobin A2: Is visual assessment reliable?

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.

This work was carried out at the suggestion of the Chairman (Professor H. Lehmann) of the expert panel on abnormal haemoglobins and thalassaemia: International Committee for standardization in Hematology.

We are grateful to the endowment funds of St Thomas’ Hospital for financial support.

References


