

Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate

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SYNOPSIS A rapid and reproducible electrophoretic method for the separation and quantitation of haemoglobins on cellulose acetate is described. The accuracy of the method and its possible sources of error are discussed. The normal range for haemoglobin A₂ by this method is 1% to 3% of the total haemoglobin concentration. Blood samples from 32 thalassaemic patients showed haemoglobin A₂ values of 3.5% to 7%.

Filter paper and starch techniques for the separation and estimation of haemoglobins are prolonged and exacting and have not been readily adopted by clinical laboratories. Simpler methods employing cellulose acetate membrane as the supporting medium were suggested by Kohn (1958 and 1960) and developed by other authors (Petrakis, Doherty, Grunbaum, and Atchley, 1962; Friedman, 1962; Afonso, 1962; Graham and Grunbaum, 1963). These have disadvantages, such as the requirement for special apparatus and staining procedures, or a failure to quantitate the A₂ fraction. Bartlett (1963) obtained reproducible values for haemoglobin A₂ in a small series of normal persons by eluting this fraction unstained.

The following method has been used in this laboratory under routine conditions and has given satisfactory and reproducible results; it is technically simple, requires no staining, and employs apparatus available in most laboratories. Results can be obtained within 90 minutes of receiving a blood sample.

APPARATUS AND REAGENTS

The apparatus requires a power pack capable of supplying direct current up to 10 mA at 200 volts, a tank suitable for electrophoresis on cellulose acetate in the horizontal plane,¹ cellulose acetate strips 5 × 12 cm.,² and a spectrophotometer with a narrow wavelength band at 413 m μ ; the Unicam SP 600 or the Hilger ultra-violet spectrophotometer are suitable instruments.

¹Shandon Scientific Company Ltd., 65 Pound Lane, Willesden, N.W. 10.

²The Oxoid Division, Oxo Ltd., Southwark Bridge Road, S.E.1.

Received for publication 27 May 1965.

Tris buffer, pH 8.9, consists of Tris-(hydroxy-methyl)-amino-methane, 14.5 g./l. (0.12M), ethylene-diamine-tetra-acetic acid, 1.5 g./l. (0.005M), and boric acid, 0.9 g./l. (0.015 M).

PROCEDURE

Blood, 2 ml., is taken into a heparinized bottle. Haemolysates are prepared by the method of Lehmann and Ager (1961). Conversion of oxyhaemoglobin to carboxyhaemoglobin is unnecessary if the solution is tested within three days.

The cellulose acetate strips are immersed in the Tris buffer for five minutes, blotted evenly between two sheets of Whatman no. 3 filter paper to remove excess moisture, and mounted horizontally in the electrophoretic tank. The strips are allowed to equilibrate in the closed unit for 10 minutes.

The qualitative separation of haemoglobins is performed on 1-2 μ l. samples of the haemolysate. The test samples and controls are applied as 1 to 1.5 cm. bands and placed 0.5 cm. on the cathode side of the mid point of the strip. Several specimens may be run simultaneously. Optimal separation occurs in 45 minutes at 20 to 25 volts/cm. with a current of 0.3 to 0.5 mA/cm. The quantitation of the haemoglobins is performed in a similar manner to the qualitative procedure with the exception that approximately 30 μ l. of the haemolysate is applied as three 5 μ l. samples to each of two cellulose acetate strips. The strips are removed after a 45-minute period of electrophoresis and cut midway between the areas of greatest concentration. The haemoglobin fractions to be quantitated, other than A₂, are placed in a plastic-capped test tube and eluted in 20 ml. of Tris buffer by rotation on a blood-mixer for 30 minutes. It is necessary to elute the A₂ fractions in 4 ml. of buffer because of the smaller quantities involved. This approximates the intensities of the solutions to be compared. The eluates are measured in a spectrophotometer at a wavelength of

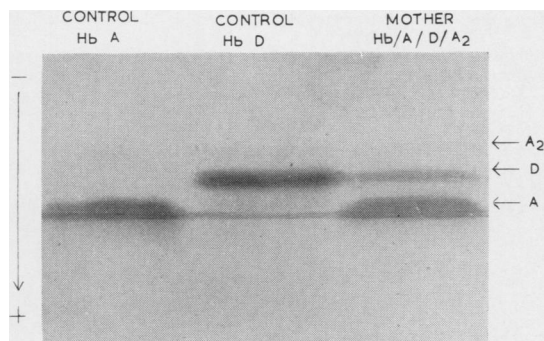


FIG. 1. Qualitative separation of haemoglobins A, D, and A_2 .

413 $m\mu$ and percentages calculated. Maximum absorption for oxyhaemoglobin solutions of A, A_2 , D, S, C, and H is recorded at this wavelength.

RESULTS AND DISCUSSION

The site of application of the haemolysate to the cellulose acetate strip is critical. At the point selected the migrating tendency of haemoglobin A towards the anode is balanced by the endosmotic flow of buffer towards the cathode. Haemoglobin A remains stationary whereas haemoglobins such as D and A_2 are carried endosmotically towards the cathode (Fig. 1). Thus haemoglobin A is prevented from migrating through the final locations of the other haemoglobins. This factor, coupled with the excellent resolving properties of cellulose acetate, gives rise to sharp zones of separation. Good results are obtained with haemoglobins A, C, D, H, S, and A_2 . Additional analytical procedures are required for the differentiation of haemoglobins with similar electrophoretic mobilities at pH 8.9, such as E and A_2 or S and D.

The range of normal values for haemoglobin A_2 for 67 healthy adults between the ages of 18 and 52 is 1% to 3% of the total haemoglobin concentration. The mean percentage is 2.0 with a standard deviation

of ± 0.51 . The mean percentage of haemoglobin A_2 is slightly lower than that reported in previous studies (Table). The disparity may be attributed to incomplete separation of haemoglobin A and A_2 by filter paper techniques because of trailing of the more rapidly migrating A fraction. In addition, the quantitative relationship between the haemoglobin concentration and uptake of stain is linear only between certain narrow ranges (Graham and Grunbaum, 1963), and their relation to the area under the optical density curve may deviate from the Beer-Lambert law when direct photometry is employed.

The reproducibility of the method was tested by 21 replicate measurements of haemoglobin A_2 on a normal sample. The results showed a mean value of 1.5% with a standard deviation of ± 0.14 . Potential sources of error arise from inaccurate cutting out of the haemoglobin bands and incomplete elution.

Blood samples from 32 patients with heterozygous thalassaemia which were examined by this method showed haemoglobin A_2 values of 3.5% to 7% (Fig. 2).

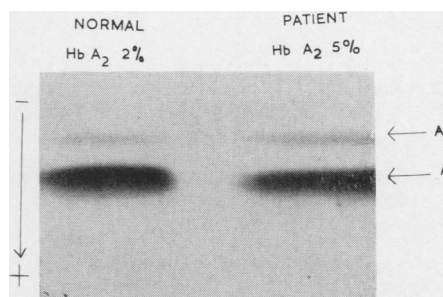


FIG. 2. Electrophoretic patterns obtained from the blood of normal and thalassaemic persons.

My thanks are due to Air Commodore W. P. Stamm, Dr. H. Bowden and Squadron Leader A. W. McCracken for encouragement and advice, to Mr. J. Watkin for

TABLE

PERCENTAGE OF HAEMOGLOBIN A_2 IN NORMAL PERSONS

Authors	Date	Technique	No.	Mean	% Range
Kunkel and Wallenius	1955	Starch block	26	2.6	1.8 to 3.5
Masri, Josephson, and Singer	1958	Starch block	230	2.6	1.2 to 3.5
Gerald and Diamond	1958	Starch block	20	2.4	1.7 to 3.1
Ibbotson and Crompton	1961	Filter paper	86	3.2	1.4 to 4.3
Hilgartner, Erlandson, Walden, and Smith	1961	Filter paper	22	9.9	5.0 to 14.3
Afonso	1962	Cellulose acetate	40	2.6	1.4 to 3.8
Petrakis <i>et al.</i>	1962	Cellulose acetate	28	3.5	1.5 to 6.1
Bartlett	1963	Cellulose acetate	17	2.5	1.8 to 3.2
Graham and Grunbaum	1963	Cellulose acetate	64	2.8	1.1 to 4.5
Present investigation	1965	Cellulose acetate	67	2.0	1.0 to 3.0

photography, and to the Director-General of Medical Services, Royal Air Force, for permission to publish.

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