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ROUTINE HbA_2 ESTIMATION BY CELLULOSE ACETATE MEMBRANE ELECTROPHORESIS°

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Abstract

Two routine procedures (A and B) for estimation of HbA_2 in hemolysate are described.

Hemolysate (Hb concentration, about 10g/dl) is prepared by the conventional technique with carbon tetrachloride for removal of erythrocyte stroma. Both of the procedures employ cellulose acetate membrane electrophoresis with tris-EDTA borate buffer solution.

A. 10 μl of hemolysate is applied to the cellulose acetate membrane (5×9 cm, pH 8.6) and an electric current (150V) is run for 90 minutes at room temperature. The electrophoresed hemoglobin stripes (A_1 and A_2) are eluted in Drabkin's solution, and the eluates are measured for absorbances in a spectrophotometer at 420 nm.

B. Aliquot of 3 μl of hemolysate is used for the sample applied to the cellulose acetate membrane. Electrophoresis is carried out at pH 8.4 (200V, 3 mA) and 6°C for 2 hours. The hemoglobin stripes thus appeared on the membrane are eluted in a buffer solution which is the same as that used for electrophoresis.

The HbA_2 content is calculated from the absorbances as shown in the equation given in the text.

INTRODUCTION

Kunkel and Wallenius[1] (1955) are credited with the discovery of HbA_2, a minor slow-moving hemoglobin component in a hemolysate of a human adult. They invented a new zone electrophoretic procedure, starch block electrophoresis, for this purpose, and found that the minor component was increased in thalassemia. This finding was confirmed by Gerald and Diamond[2] (1958) three years afterwards. However, on account of the necessity of relatively complicated assembly of apparatuses and

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manipulations for starch block electrophoresis, estimation of HbA_2 in hemolysate had not been introduced into the routine hematological services in laboratories until 1959, when Craddock-Watson, Fenton and Lehmann[3] succeeded in the separation of HbA_2 stripe from the band of Hb A_1, the major hemoglobin component, by paper electrophoresis. Ibbotson and Crompton[4] (1961) obtained, by this technique, a HbA_2 content in hemolysate of 5.1-8.6 % for thalassemia minor in contrast to that of 1.4-4.3 % for normal adults.

Since that time HbA_2 has excited much interest of hematologists and clinical pathologists who are engaged in the study of hemoglobinopathy. A variety of methods including chromatographies[5] (Amberlite IRC 50, DEAE cellulose, DEAE Sephadex) and electrophoreses (starch gel[6], agar gel[7]) have been devised for HbA_2 estimation. In our experience, cellulose acetate membrane electrophoresis (pH 8.4 or 8.6) has proved to be the most convenient method which gives satisfactorily exact and accurate values, if the hemoglobin stripes (A_1 and A_2) are cut out, and eluted in Drabkin's solution or Tris-EDTA-borate buffer solution (pH 8.6) to be measured for their absorbances at 415 nm (Soret band). Densitometry of the electrophoresed hemoglobin stripes stained with dyes (Ponceau S, Amido Black 10-B) or benzidine reaction was not reliable.

This paper aims to describe the two procedures of our cellulose acetate membrane electrophoresis (at room temperature and at 6°C) for the estimation of Hb A_2.

MATERIALS AND METHODS

A. *Reagents*

(1) Tris-EDTA-borate buffer solution (pH 8.6)

27.5 g of tris-hydroxy-methyl-aminomethane, 3.5 g of ethylene-diamine tetra-acetic acid and 4.5 g of boric acid are dissolved in H_2O and made to total volume of 1500 ml.

(2) Tris-EDTA-borate buffer solution (pH 8.4)

10.2 g of tris-hydroxy-methyl aminomethane, 0.6 g of ethylene-diamine tetra-acetic acid and 3.2 g of boric acid are dissolved in distilled water and made to volume of 1000 ml.

(3) Drabkin's solution

100 mg of potassium ferricyanide $K_3Fe(CN)_6$, 25 mg of potassium cyanide KCN, and 500 mg of sodium bicarbonate $NaHCO_3$ are dissolved in H_2O to make the total volume up to 500 ml.

B. *Apparatuses*

(1) Cellulose acetate membrane

Membranefilter Gm BH of Sartorius was cut to obtain 5×9 cm or 7×20 cm rectangular pieces.

(2) A set of cellulose acetate membrane electrophoresis apparatus.

This is supplied by the Fuji-Riken Co. It has the electric capacity of 300 V and 100 mA. Toyo filter paper No. 1 or Whatman 3 MM paper is cut and folded in double to use as wicks.

(3) Kirk's ultramicropipet (for 3 μl or 10 μl).

(4) Other apparatuses

Test tubes (for eluation of hemoglobin stripes of cellulose acetate membrane after electrophoresis), test tube racks, filter papers, pipets, scissors, etc.

C. Procedures

(I) Electrophoresis at room temperature and eluation with Drabkin's solution

(*1*) *Preparation for electrophoresis:*

A cellulose acetate membrane (5×9 cm) is immersed in a buffer solution (pH 8.6) which has been diluted to two-folds with H_2O to be wetted uniformly, put between filter papers to remove excessive moisture, and applied so as to span between the anode and the cathode of an electrophoretic apparatus; then electrode baths are filled with the buffer solution (pH 8.6 without dilution), and an electric current of 150 V is run for several minutes.

(*2*) *Application of hemolysate:*

10 μl. of a hemolysate (Hb concentration, about 10 g/dl) is applied to a position 3 cm apart from the cathodal end of the cellulose acetate membrane piece by an ultramicropipet. The electrophoretic apparatus is covered tightly with a rid.

(*3*) *Applying an electric current:*

An electric current of 150 V is applied for 90 minutes. The stripes of HbA_1 and HbF and that of HbA_2 are distinguishable macroscopically at the end of this time.

(*4*) *Elution:*

The electric source is shut, and the cellulose acetate membrane is taken out. From it (1) area containing the stripe of HbA_1+HbF and (2) that containing the stripe of HbA_2 are separately cut out, and the former piece is put in a test tube containing 60 ml of Drabkin's solution (1), while the latter into another test tube containing 30 ml of Drabkin's solution (2) to be eluated.

(5) *Colorimetry:*

A filter of 420 nm is inserted to a photoelectric colorimeter, and setting the optical density to 0 with Drabkin's solution the optical densities of the solutions of the test tubes (1) and (2), namely OD_1 and OD_2, are read.

(6) *Calculation:*

The HbA_2 content of hemolysate is calculated by the following equation:

$$HbA_2\ (\%) = \frac{OD_2}{2 \times OD_1 + OD_2} \times 100$$

(II) *Electrophoresis at 6°C and eluation with tris-EDTA-borate buffer solution*

(1) *Preparation for electrophoresis:*

Cellulose acetate membrane (7×20 cm) is soaked in the tris-EDTA-borate buffer solution (pH 8.4) for sufficient time, put and pressed between two sheets of filter papers to remove excess of buffer solution and set to the electrophoretic apparatus as described in (I).

(2) *Application of hemolysate:*

3 μl of hemolysate is applied to a position 1.5 cm apart from the cathodal end of the membrane by an ultramicropipet, in duplicate, making two separate rectilinear lines 1.5 cm in length. The electrophoretic apparatus is covered tightly with a rid.

(3) *Electric current:*

200 V and 3 mA, at 6°C for 2 hours.

(4) *Eluation:*

The hemoglobin stripes, HbA_1 and HbA_2, are cut out of the membrane. HbA_2 piece is put into a centrifuge tube containing 3 ml of tris-EDTA-borate buffer solution (pH 8.4), and HbA_1 piece to 30 ml of the same buffer solution in a beaker. The centrifuge tube is subjected to centrifugation (1500 RPM for 5 minutes).

(5) *Colorimetry:*

The eluates are measured for the absorbances OD_1 (for HbA_1) and OD_2 (for HbA_2) at 415 nm in a spectrophotometer against the blank (tris-EDTA-borate buffer solution of pH 8.4).

(6) *Calculation:*

$$HbA_2\ (\%) = \frac{OD_2}{10 \times OD_1 + OD_2} \times 100$$

RESULTS AND DISCUSSION

In these methods, it is assumed that if HbA_1 and HbA_2 are equal in concentration, they will show the same absorbances in the Soret region, either in oxygenated form or cyanmethemoglobin form. This assumption will be granted in approximation.

HbA_2 content was measured in 427 normal subjects by the procedure (I) (eluation in Drabkin's solution). The results are shown in Figure 1, in which is seen a normal distribution of mean value $m=2.52 \pm SD\ 0.52\%$. Therefore, the normal range is from 1.48 to 3.56 %. Procedure (II) (eluation in Tris-EDTA-borate buffer) gave a normal range between 2.6 and 3.3 % with a mean value of 2.9 % when 50 normal subjects are used as materials. At first glance the normal range may seem to be narrower in procedure (I), but it will be germane to consider that these two procedures are almost the same in the appraisal of normal range of HbA_2, because the subjects examined by the procedure II was too small in number.

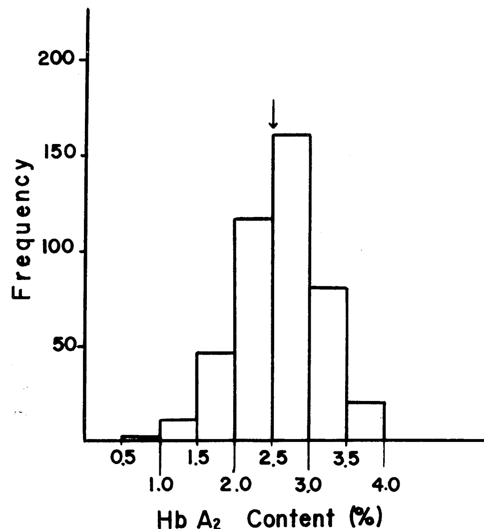

Fig. 1. Distribution of the A_2 content of normal subjects

The electrophoretic methods which have hitherto been invented for the estimation of HbA_2 content are classified into two groups: (1) densitometry of the hemoglobin stripes directly or after staining, and (2) colorimetry of the hemoglobin stripes after eluation. In Table 1 are listed the normal range of HbA_2 content reported by various authors who employed a variety of methods, including electrophoreses and chromatographies.

TABLE 1.
Percentages of HbA_2 in normal adults

Method	Mean	Range	Author		Ref.	
Starch block	2.6	1.8-3.5	Kunkel and Wallenius	1955	1	*
Cellulose acetate	2.9	2.6-3.3	Ueda and associates	1975		*
CM cellulose column	2.2	1.8-2.5	Muller and Pik	1962	16	*
DEAE sephadex column	2.6	1.8-3.2	Jonxis and Huisman	1968	5	*
Starch gel	2.3	1.5-3.1	Sunderman	1963	6	* and **
Agar gel	6.7	4.1-9.7	Yakulis and associates	1960	7	**
Filter paper	3.2	1.4-4.3	Ibbotson and Crompton	1961	4	**
Acrylamide gel	2.9	1.4-4.4	Ferris and associates	1965	8	**
Cellulose acetate	3.5	1.5-6.1	Petrakis and associates	1962	1	**

The percentages of HbA_2 in normal adults as determined by different methods. An asterisk (*) indicates the method by colorimetry of the hemoglobin stripes after eluation. Double asterisk (**) indicates the method by densitometry of the hemoglobin stripes directly or after staining.

Starch block electrophoresis, CM cellulose column chromatography and DEAE sephadex column chromatography will be accepted as standard methods for the estimation of HbA_2 at the present time. Scrutiny of Table 1 makes us to notice that the densitometry of hemoglobin stripes directly or after staining shows a normal range of HbA_2 which is significantly higher than that given by the eluation of the hemoglobin stripes followed by colorimetry, and that the latter (eluation technique) is in good agreement with standard methods. Our two procedures belong to the eluation technique, and in fact they give the normal range almost identical with that obtained by starch block electrophoresis. Erroneously high normal value by densitometry of hemoglobin stripes directly or after staining will be ascribed to the interference caused by the supporting media used for electrophoresis.

The precision and recovery of HbA_2 estimation by the procedure (I) is summarized in Table 2. The coefficient of variation (CV) was 0.01 % in the procedure II in 50-plicate estimation of the same hemolysate sample (HbA_2 content, 3.38 %).

The recovery rate and the coefficient of variation varied in the procedure (I) depending on the HbA_2 content of hemolysates (Table 2); with lower contents (1-3 %), HbA_2 was recovered a little in excess and CV was larger (about 10 %), while with higher contents (about 10 %), it was not recovered up to 100 %, but CV was smaller (3-4 %). Accordingly the precision is low with the HbA_2 content less than 1 %,

TABLE 2.
Precision* and recovery of HbA_2 estimation

Sample**	HbA_2 content		Recovery rate (%)	CV (%)
	Expected	Actual		
1	0.23	0.24	104.3	10.6
2	0.46	0.47	102.2	14.6
3	0.93	0.79	85.1	10.5
4	1.49	1.30	87.5	4.1
5	1.85	1.70	92.1	2.9
6	2.32	2.20	94.8	2.7
7	2.78	2.63	94.6	2.7
8	3.70	3.40	92.2	2.9
9	4.63	4.43	95.7	3.7
10	9.26	8.09	87.8	3.1

* Ten-time-repeated estimations
** Artifical hemolysates composed of HbA_1 and HbA_2 separated and purified by DEAE sephadex column chromatogrpahy

TABLE 3.
Daily measurement of HbA_2 content of the same hemolysates for 10 days

Sample	Mean HbA_2 content	CV (%)	Mean HbA_2 content corrected	CV (%) corrected
1	2.67*	5.62*	2.79	2.07
2	2.75*	9.46*	2.94	2.25
3	2.58	2.55	——	——
4	3.00*	4.95*	3.02	3.11
5	2.82	4.83	——	——
6	3.11*	3.79*	3.18	1.40
7	3.00*	4.59*	3.07	3.10
8	2.89*	5.58*	3.00	2.30
9	2.78*	7.39*	2.86	2.25
10	2.88*	5.36*	2.92	4.74
	estimated as such		methemoglobinized sample was excluded	

* On one of the days between the seventh and the tenth a methemoglobinized sample was encountered

and with higher contents the HbA_2 can be measured with a precision of CV=3 %, generally. Similar aspects will be seen also for the procedure (II).

A hemolysate sample was divided into ten specimens, which were preserved in a refrigerator, and each one of them was in turn measured daily by the procedure (I) over the period of ten days. The results (Table 3) showed that the content could be measured with a day-to-day variation of CV less than 3% around the mean value, but when the specimens became methemoglobinized partially, the CV was larger. The procedure (II) is not adequate for this kind of trial.

The HbA_2 content of a hemolysate can be estimated with the variation of $\pm$ SD 3% from the true value by the two procedures described in the present paper, if the sample is stored in a refrigerator and is not aged too much, namely within 6 days after its preparation.

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