Kawasaki Med. J. Vol. 4 : No. 2, 139-142, 1978

BRIEF NOTE

A SIMPLE METHOD FOR THE DETERMINATION OF HEMOGLOBIN A_2 BY MEANS OF ISOELECTRIC FOCUSING ON POLYACRYLAMIDE GEL

Accepted for Publication on July 11, 1978

Quantitative determination of hemoglobin A_2 and hemoglobin F is one of the important tests for the diagnosis of the β thalassemia syndrome. Generally, estimation of hemoglobin A_2 have been carried out by cellulose acetate membrane electrophoresis[1] and micro-column chromatography using DEAE-cellulose (DE-52)[2,3]. In this paper an successful use of isoelectric focusing on pH gradient 6-9 of polyacrylamide gel for hemoglobin A_2 assay will be described.

The pH gradient 6-9 of polyacrylamide gel was prepared as follows: The mixture of acrylamide-Bis solution (3.0 ml), which was prepared by dissolving acrylamide monomer (30 g) and N,N′-methylenebisacrylamide (0.8 g) in water and made to 100 ml of final volume. To this solution were added 20.8 percent aqueous solution of sucrose (11 ml), ampholine pH 3.5-10 (0.15 ml), ampholine pH 7-9 (0.60 ml) and water (3.0 ml), mixed completely and deaerated, followed by addition of 0.004 percent aqueous riboflavin solution (0.1 ml), N,N,N′,N′-tetramethylethylenediamine (0.05 ml) and 10 percent aqueous ammonium persulfate solution (0.05 ml). The mixture thus prepared was introduced into the narrow space (1 mm in thickness) which is formed between the two sheets of glass plate (130 mm×180 mm). A small amount of water is overlayed above the mixture and the glass plate assembly is illuminated by ultraviolet light for 3 hours to polymerize the mixture. At the end of the time one of the glass plate is removed. A rectiangular acrylamide gel plate (160 mm×110 mm×1 mm) lying on another glass plate is obtained. A long rectiangular strip (6 mm×160 mm×0.75 mm) of a thick filter paper was laid on the middle line of the gel surface to make it serve as the mat of the anode of electrophoresis, while at the bilateral edges of the same gel are placed the filter paper trips of the same shape and size for the purpose of the cathodes (Figure). The filter paper strips of the anode is moistened with 0.02 M aqueous solution of phosphoric acid while those of the cathodes are imbibed

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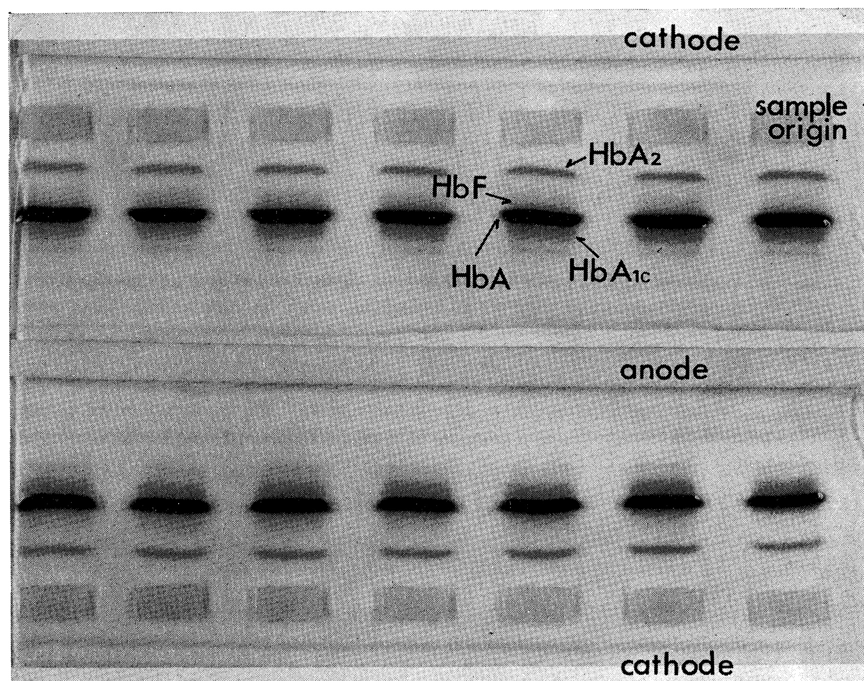

Figure. Isoelectric focusing pattern of 14 normal hemolysates on the polyacrylamide gel plate (160×110×1 mm)

with a sufficient amount of 20 percent aqueous ethylenediamine solution. Rectiangular filter papers (5 mm × 10 mm) are soaked with individual hemolysates about 10 μl aliquots are absorbed, and placed on the gel surface with 1 cm interspace along the rectilinear line 1 cm apart from the cathode lines. Fourteen hemolysate samples are applied on the get plate. The gel plate is placed in a cooling chamber at 8°C. The anode and cathodes are connected to the electric source and an electric current of 200 V/32 cm is run for an hour and then current is raised to 400 V/32 cm and stabilized at this level for the next one hour. The pH gradient is rectilinear from 6 to 9. At the end of the time the acrylamide gel band containing hemoglobin A_2, and the portion of those possessing hemoglobin A–A_{1c} and hemoglobin F, are enucleated separately from the gel plate with a cutter, and the relevant bands are put into two tubes (A_2 and A–A_{1c}+F) containing 4 ml and 10 ml of 1/15 M phosphate buffer (pH 7.4) individually to be elute completely by occasional mixing by inversion, until the gel bands becomes transparent, spending three hours at least. The absorbances of these eluates are measured at 415 nm

against water as reference and the contents of hemoglobin A_2 (%) are calculated from the equation as follows:

$$\text{Hemoglobin } A_2(\%)=\frac{A_2OD}{A_2OD+2.5\times(A-A_{1c}+F)OD}\times 100$$

The fluctuation of the values of hemoglobin A_2 content (%) of the same blood samples by repeated determinations on different days is within $\pm 0.2\%$.

When this quantitative analysis of the hemoglobin A_2 by isoelectric focusing was devised, the author took advantage of the fact that hemoglobin A_2 was seldom contaminated by the other hemoglobins, e. g. hemoglobin F and/or hemoglobin A, since hemoglobin A_2 possessed highest pI values of all normal hemoglobins and thus it could be isolated completely. However, in the aged hemolysate methemoglobin (or half-methemoglobin) was produced a little, and, therefore, the accuracy of quantitation tended to be lowered. On the cellulose acetate membrane electrophoresis, which has been employed for the determination of hemoglobin A_2 for a long time to date, tailing is unavoidable and, therefore, accuracy is impaired. The DE-52 column chromatography is not free from problems. The hemoglobin components (A_2, $A-A_{1c}$ and F) are absorbed and eluted or not eluted appropriately on the column package, depending on the delicate adjustment of pH of the developers. This affects the precision of hemoglobin A_2 estimation.

In our experience the isoelectric focusing method which has been presented in this paper is simple and precise. Fourteen blood samples are disposed with at a time on a gel plate. This method is recommended as a useful tool for clinical diagnosis.

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