วารสารเภสัชศาสตร์เชียงใหม่
Bulletin of Chiang Mai
Associated Medical Science

Vol. 9 No. 3
September 1976

DICHLOROPHENOLINDOPHENOL (DCIP) PRECIPITATION TEST

By

Panja Kulapongs, M.D.
Torpongs Sanguansermsri, M.D.
Suchada Tawarat, B.Sc.
Greg Mertz, M.D.

ABSTRACT

The blue dye, 2,6 dichlorophenol-indophenol (DCIP) has been used successfully in the screening test and the quantitative assay of erythrocyte G-6PD, Glutathione reductase (GSSG-R). and 6-phosphogluconic dehydrogenase (6-PGD) activities. It was later noticed that when DCIP was added to the hemoglobin E hemolysate the gross turbidity resulting from hemoglobin precipitation occurred. Based on this unusual phenomenon, the DCIP precipitation was designed. The authors investigated the sensitivity of this test as a screening for the presence of the two most common unstable hemoglobin mutations in Thailand. Three characteristic absorbancy curves at 800 mu due to the presence of hemoglobins E, H, and A/F are established. This simple spectrophotometric method is of particular useful for the mass screening test. The presence of either hemoglobin E or H can be detected within 60 to 90 minutes and its exact nature can be determined within 3 hours.

INTRODUCTION

The knowledge that the blue dye, 2,6 dichlorophenolindophenol (DCIP) could be decolorized (reduced) by reduced glutathione without artificial electron carrier has been used as the basis of the quantitative assay

and of screening test for erythrocyte G-6-PD glutathione reductase (GSSG-R) and 6-phosphogluconic dehydrogenase activities (1-5). In the course of the investigations Frischer et al noticed that the activities of these enzymes were not altered by the presence of Hb. E but the gross turbidity as the results of hemoglobin precipitation occurred (4 5). The turbidity in the presence of Hb. E did not interfere with the color detection of activities of G-6-PD or GSSG-R but did result in increased absorbancies at 660 mu. In contrast, hemolysates from individuals with Hb. AS, SS, SC, CC, AC, AD and beta thalassemia with increased Hb F did not give unusual results(6). These observations indicated that Hb. E is an oxidatively unstable hemoglobin(6). It is speculated that this instability of Hb. E resulting from interference by the mutation with the dimeric contact between the alpha and beta chains.

It was felt that this unusual phenomenon which appears to be a result of direct interaction between DCIP and Hb. E may be useful in the detection of the presence of Hb. E. in our population. We are reporting our experience with a simple spectrophotometric procedure based on this phenomenon in the detection of the presence of hemoglobin E in our clinic.

MATERIALS AND METHOD

Blood samples were obtained from 20 normal adults, 8 children and 2 adults with beta thalassemia Hb. E disease, 3 adults with Hb.AE, 5 children with beta thalassemia major, 3 adults with beta thalassemia major, 3 adults with beta thalassemia trait (AF), 3 children with alpha thalassemia Hb.H and 3 adults with alpha thalassemia trait. Five milliliter of blood sample was added to 1 ml. of ACD solution and kept in 4°C refrigerator to be studied within 60 minutes.

WORKING REAGENT. In a 500 ml. volumetric flask add 4.36 gm. (7.2×10^{-2}M) of Trizma base (Sigma Chemical Co.); 2.68 gm. of EDTA $Na_2 . 2H_2O$ (Fisher Scientific Co.); 27.6 mg (1.9×10^{-4}M) of dichlorophenolindophenol (Sigma Chemical Co.); and 50 mg. of Saponin (Fisher Scientific Co.). Add distilled deionized water, adjust to pH 7.5 at room temperature with HCl and add water to the final volume of 500 ml.

PROCEDURE. Blood sample is centrifuged at 80 x G for 10 minutes at room temperature. Five milliliter of the working reagent is transferred into a 10 x 75 mm. glass tube (cuvette) followed by 0.020 ml. of sedimented red cells from the bottom of the centrifuged blood sample tube, mixed by inversion, allowed to stand for 10 minutes at room temperature before being placed in a 37°C water bath without further mixing or agitation. At a specific time interval the cuvette is placed into a spectrophotometer (Coleman Jr. II, Model 6/20) and the absorbancy at 800 mu against a water and buffer blank is recorded.

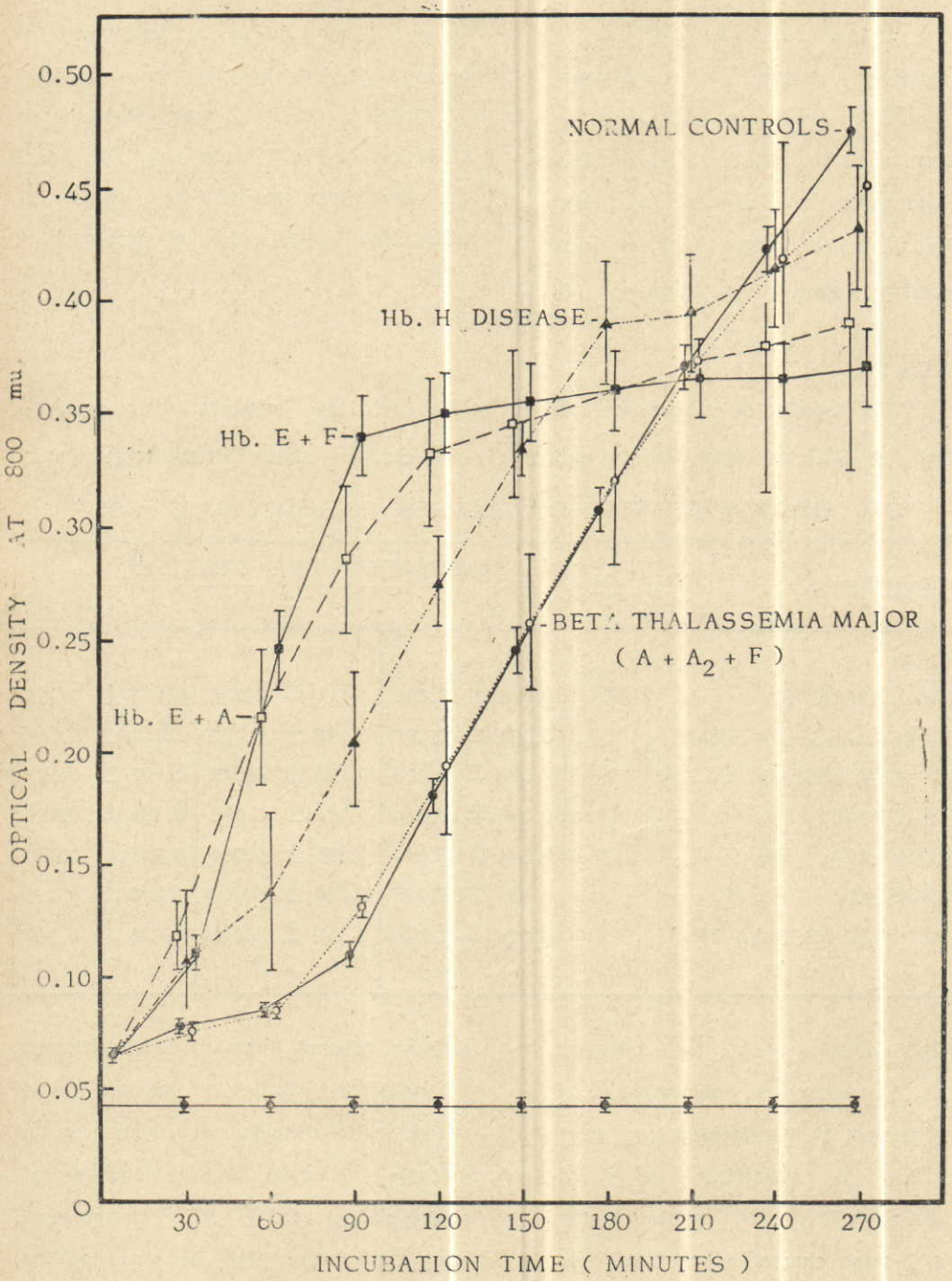
RESULTS :

The results of absorbancy change of the reaction mixtures (hemolysates + DCIP) at 800 mu from various groups of subjects are shown in the table below.

O.D. READINGS OF REACTION MIXTURES OF HEMOLYSATE + DCIP

SUBJECTS	NO.	INCUBATION TIME (MINUTES)		
		60	90	120
NORMAL ADULTS	20	0.085 ± 0.006	0.111 ± 0.026	0.179 ± 0.035
BETA THALASSEMIA TRAIT	3	0.084 ± 0.007	0.128 ± 0.022	0 233 ± 0.041
BETA THALASSEMIA MAJOR	5	0.084 ± 0.005	0.133 ± 0.043	0.193 ± 0.067
Hb. E TRAIT	3	0.216 ± 0.054	0.286 ± 0.058	0.332 ± 0.059
THALASSEMIA Hb. E	10	0.244 ± 0.054	0.339 ± 0.055	0.349 ± 0.052
THALASSEMIA Hb. H	3	0.137 ± 0.065	0.204 ± 0.052	0.276 ± 0.036
ALPHA THALASSEMIA TRAIT	3	0.089 ± 0.020	0.112 ± 0.028	0.176 ± 0.057

The variation due to different degrees of anemia is eliminated by the utilization of sedimented red cells (which has the hematocrit value of approximately 72 %). As shown in the Figure I, three characteristic absorbancy curves due to the presence of Hb. E, Hb. H and Hb. A and/or F are established. At the present time the O.D. reading of more than 0.108 at minutes and 0.168 at 90 minutes of incubation is strongly indicate the presence of hemoglobins E and H (95 % confidence). The exact nature can be obtained by observing the absorbancy curve 2-4 hours later.


NORMAL CONTROLS
Hb. H DISEASE
Hb. E + F
Hb. E + A
BETA THALASSEMIA MAJOR
(A + A2 + F)
OPTICAL DENSITY AT 800 mu
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
30
60
90
120
150
180
210
240
270
INCUBATION TIME (MINUTES)

COMMENT

The absorbancy at 800 mu is used because at this wavelength the spectral contributions of both hemoglobin and DCIP itself are minimal (6). The increased absorbancy in this test system is paralleled by a decreased residual hemoglobin concentration in the centrifuged supernate. In a given blood sample, the occurrence and degree of turbidity is reproducible and is not abolished by thoroughly destromatizing the hemolysate. The change in absorbancy is directly proportional to hemoglobin concentration (at a given DCIP concentration) and to the DCIP concentration when that of hemoglobin is kept constant (6). Precipitation is proportional to temperature (varied between 4°C and 45°C), inversely proportional to pH (varied between 6.0 and 8.0), and somewhat more marked with 7.5×10^{-2} M Tris buffer than in equimolar phosphate buffer (both at 37°C, and pH 7.0). The washed precipitate which is insoluble in water could be dissolved in 0.1N HCl and showed the Soret absorption bands of porphyrins with additional shoulders at 541,576, and 630 mu.

Three characteristic absorbancy curves are established. The normal individuals and those with beta thalassemia disease or trait exhibited a flat initial curve until 90 minutes of incubation when the curve rose sharply during the following hours (see Figure 1), and may be called A/F curve. The presence of hemoglobin E in the hemolysate is characterized by a sharp rise in the absorbancy curve reaching almost maximum or maximum reading at 90 minutes before leveling off (E curve). The presence of hemoglobin H is characterized by the moderately high initial reading then followed by continuous rise of absorbancy curve similar to those of A/F curve.

It is evidence that this simple test will be useful as the rapid screening test for the presence of hemoglobins E and H, the most common unstable hemoglobin mutations in Thailand. At presence, we consider the initial absorbancy reading of higher than 0.108 at 60 minutes of incubation or 0.168 or more at 90 minutes is the indication of the presence of either hemoglobin E or H. By following

the individual absorbancy curve during the next few hours, its exact nature can be determined as hemoglobin E or H. The further modification of this test to serve as a rapid semiquantitative test is under investigation.

ย่อเรื่อง

ในการตรวจทั้งแบบ Screening และ Quantitative assay เพื่อหาปริมาณของ Erythrocyte G-6-PD, Glutathione reductase (GSSG-R) และหา activity ของ 6-phosphogluconic dehydrogenase (6-PGD) ปรากฏว่า สีที่ใช้ในการตรวจและให้ผลดีคือ blue dye, 2,6 dichlorophenolindophenol (DCIP) ต่อมาได้พบว่า เมื่อเติม DCIP ลงไปใน Hb. E hemolysate จะเกิดความขุ่นอันเนื่องมาจากการตกตะกอนของ Hb. และอาศัยปรากฏการณ์อันนี้ คณะผู้ทำการศึกษาและวิจัยชุดนี้ จึงได้เตรียมการตรวจเกี่ยวกับการตกตะกอนของ DCIP และได้ตรวจหา Sensitivity ของวิธีที่ใช้ตรวจ ซึ่งเป็นการตรวจแบบ Screening เพื่อหา unstable hemoglobin mutations สองชนิดที่พบได้บ่อยที่สุดในประเทศไทย

จากผลการตรวจพบ characteristic absorbancy curves 3 แบบที่ 800 mu อันเนื่องมาจาก Hb.E, H และ A/F ซึ่งการตรวจแบบง่าย ๆ โดยวิธี Spectrophotometry เช่นนี้ จะเป็นประโยชน์ต่อการตรวจที่มีปริมาณการส่งตรวจมาก ๆ โดยสามารถตรวจพบ Hb. E. หรือ Hb. H ภายในเวลา 60–90 นาที และสามารถบอก nature ที่แท้จริงของ Hb. เหล่านี้ได้ภายในเวลา 3 ชั่วโมง

REFERENCES

1. Ells, H.A., and Kirkman. H.N.: A colorimetric method for assay of erythrocytic glucose-6-phosphate dehydrogenase Proc. Soc. Exp. Biol. Med. 106: 607, 1961.

2. Bernstein, R.E.: A rapid screening dye test for the detection of glucose-6-phosphate dehydrogenase deficiency in red cells. Nature 94: 192, 1962.

3. Kaplan, T.C.: Electrophoretic study of glutathione reductase in human erythrocytes and leukocytes. Nature 217: 256, 1968.

4. Frischer, H., Bowman, J E., Carson, P.E., Rieckmann, K.H., Willerson, D., Jr., and Colwell, E.J.: Erythro-

cytic glutathione reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconic dehydrogenase deficiencies in populations of the United States, South Vietnam, Iran, and Ethiopia. J. Lab. Clin. Med. 81 : 603, 1973.

5. Frischer, H., Carson, P.E., Bowman, J.E., and Rieckmann, K.H.: Visual test for erythrocytic glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, and glutathione reductase deficiencies. J. Lab. Clin. Med. 81 : 613, 1973.

6. Frischer, H., and Bowman, J.E.: Hemoglobin E, an oxidatively unstable mutation. J. Lab. Clin. Med. 85 : 531, 1975.

ท่านสมาชิก

ที่หมดอายุการบอกรับวารสาร โปรดต่อสมาชิกภาพ