AN IMPROVED SINGLE-STEP SCREENING METHOD FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

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Abstract: We have established a new simple and rapid screening method for glucose-6-phosphate dehydrogenase (G6PD) deficiency. The principle of this method is the formation of blue formazan with the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) produced by G6PD absorbed on a DEAE-Sephadex anion exchanger. The whole procedure is performed in a microcentrifuge tube and it can be completed in less than 30 min without any special equipment other than micropipettes. Our method is particularly suitable for field detection of G6PD-deficient subjects prior to administration of primaquine in situ.

Key words: G6PD deficiency, screening method, acute hemolytic anemia, primaquine

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the most common hereditary disorders, which is prevalent in malaria endemic areas, probably because G6PD-deficient erythrocytes are resistant to Plasmodium falciparum infection (Luzzatto and Mehta, 1995). G6PD deficiency may cause various types of hemolytic anemia, most typically an acute hemolytic attack after taking certain oxidative drugs such as primaquine.

Primaquine has been widely used for the radical treatment of Plasmodium vivax malaria. In addition, its gametocytocidal action is effective to cut the transmission of Plasmodium falciparum gametocytes in endemic areas (Matsuoka et al., 1987; Doi et al., 1989). Primaquine-induced hemolytic crisis in G6PD-deficient individuals is a serious problem in the chemotherapeutic malaria control activities. Thus, it is important to screen out G6PD-deficient individuals before staring the operation (Ishii et al., 1994). Malaria control activities are often carried out in the field where no electricity is available. In addition, many patients travel over great distances from their villages and both diagnosis and initial administration of primaquine must be completed on the same day. Under such conditions, it is necessary to complete the whole screening procedure from collecting blood to interpreting the results in less than one hour without any electrical equipment. Current screening tests for G6PD deficiency including a fluorescent method (Beutler, 1966; Beutler and Mitchell, 1968) and previous formazan methods (Fairbanks and Beutler, 1962; Fujii et al., 1984) do not fully meet the requirements.

We describe here an improved single-step formazan method suitable for rapid screening for G6PD-deficient subjects in the field. Our procedure can be completed within 30 min without any special equipment.

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate (NADP⁺) were purchased from Boehringer-Mannheim (Germany) and DEAE-Sephadex A-50 was from Pharmacia (Uppsala, Sweden). 3 (4,5 Dimethylthiazolyl 1-2) 2,5 diphenyltetrazolium bromide (MTT) was from Dojin (Kumamoto, Japan) and phenazine methosulphate (PMS) was from Sigma (St. Louis, MO). Other reagents were of analytical grade.

Preparation of mixtures

DEAE-Sephadex A-50 was equilibrated in 0.1 M
Table 1 Reaction mixtures

<table>
<thead>
<tr>
<th></th>
<th>Standard method (µl)</th>
<th>GSSG method (µl)</th>
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<tbody>
<tr>
<td>DEAE-Sephadex</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Substrate mix</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>MTT-PMS mix</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>4.8mM GSSG</td>
<td>—</td>
<td>200</td>
</tr>
<tr>
<td>H₂O</td>
<td>200</td>
<td>—</td>
</tr>
<tr>
<td>Whole blood</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Tris·HCl, 10 mM MgCl₂, pH 6.5. The substrate mix contained 5 mM G6P, 0.4 mM NADP⁺, 0.2 % Saponin in H₂O and the MTT-PMS mix contained 0.025 % of each reagent in H₂O.

Procedure

All the reactions were carried out in a 1.5 ml microcentrifuge tube (Eppendorf) at room temperature. A tube containing 200 µl each of DEAE-Sephadex A-50 gel, the substrate mix, the MTT-PMS mix and distilled water was prepared (Table 1). The reaction was started by adding 5 µl of whole blood to the tube and mixing by shaking several times. The tube was then left to stand. A gel bed formed immediately by natural sedimentation and was clearly separated from the upper reddish aqueous layer. After 20 min incubation, the development of blue color on the gel with patient's blood was compared with that with control blood.

Results

Fig. 1 shows the blue color development. With normal control samples, color development was apparent after 20 min incubation and the intensity reached a maximum after 40 min. We tested several G6PD-deficient samples with various residual activities. Samples with less than 30% residual activities showed very slow color development, and could easily be distinguished from normal samples until after 24 h of incubation. However, G6PD-deficient samples with higher residual activities showed more rapid color development and it was difficult to differentiate them from normal samples after more than 12 h incubation. Adding oxidized glutathione (GSSG) to the reaction mixture ("GSSG method" in Table 1) reduced such ambiguity in interpreting the results after prolonged incubation periods (Beutler and Mitchell, 1968). The optimal amount of blood for addition to the reaction mixture was 5-10 µl. Although amounts up to 20 µl were acceptable, adding more blood caused difficulty in interpreting the results.

We also found that dried blood blotted on regular filter paper or a cation-exchange cellulose paper (Whatman, P 81) could be used as samples. After adding a piece of filter paper with dried blood to the reaction mixture, the tube was shaken several times and stood for 5 min to make remove blood from the paper. The tube was then inverted to make the filter paper attach on the reverse side of the cap and was then kept upright as usual. When testing a large number of samples, the reaction could be done in 96-well microtiter plates in place of microcentrifuge tubes using 1 µl of blood with a one tenth reduction in reaction volume.

The stability of the prepared mixtures was examined. After one year storage of DEAE-Sephadex at room temperature, the MTT-PMS mix kept in a dark bottle at 4°C, and the substrate mix at –20°C with frequent freezing and thawing, respectively, we found no changes in their efficiency. The substrate mix and the MTT-PMS mix were also stable at room temperature for several days.

![Figure 1 Blue color development in reaction tubes with blood samples from a normal control subject (Ct), a G6PD-deficient patient (Pt) and a heterozygous female (Ht).](image-url)
DISCUSSION

A number of methods for rapid diagnosis of G6PD deficiency have been described (Beutler et al., 1955; Brewer et al., 1960; Bernstein, 1962; Fairbanks and Beutler, 1962; Beutler, 1966; Beutler and Mitchell, 1968; Fujii et al., 1984). Among these, a fluorescent method (Beutler, 1966; Beutler and Mitchell, 1968) and some formazan methods (Fairbanks and Beutler, 1962; Fujii et al., 1984) have been adopted. The spot test developed by Beutler and Mitchell (1968), which depends upon the fluorescence of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as an indicator of G6PD activity, was recommended as a standard screening method by the International Committee for Standardization in Hematology (Beutler et al., 1979). Although the procedure is reliable, simple and can be completed in less than 30 min, it requires ultraviolet (UV) light to examine the results, which might be a significant drawback in applying the procedure to the rapid detection of G6PD-deficient subjects in the field.

The tetrazolium dye MTT forms blue colored formazan when reduced in the presence of a hydrogen carrier such as PMS. This reaction can be used as an indicator of G6PD activity by monitoring production of the potent reducer NADPH. Although the principle is straightforward, the reaction cannot be applied directly to blood samples because hemoglobin reacts nonspecifically with MTT and its dark red color strongly interferes with interpretation of the result. Several attempts have been made to overcome this problem, including absorption of either G6PD on anion-exchange cellulose paper (Fairbanks and Beutler, 1962) or hemoglobin on cation-exchange cellulose paper (Fujii et al., 1984). Although these methods have the advantage of requiring no UV light when examining the results, the procedures might be too laborious and time-consuming to be carried out in the field.

Our present method also depends on formazan formation. G6PD in blood is absorbed on DEAE-Sephadex beads and separated from hemoglobin in the aqueous layer by natural sedimentation. Since the reaction progresses only in the gel, the nonspecific reaction of hemoglobin with MTT and the interference in the color development can be kept at a minimum. The bluing of the gel is apparent after 20 min incubation with normal samples and can readily be distinguished from that with G6PD-deficient samples. The whole single-step procedure can be completed in a microcentrifuge tube in less than 30 min without any special equipment other than micropipettes. In comparison with other procedures, our method has the great advantage of its rapidity and simplicity with similar reliability, although it might not be very useful for some genetic surveys which require dealing with thousands of samples at a time. These features make our method particularly suitable for field detection of G6PD-deficient subjects prior to administration of primaquine in situ as well as for ordinary laboratory tests.

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REFERENCES