Methaemoglobin Reduction Test *

A New, Simple, in Vitro Test for Identifying Primaquine-Sensitivity

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The 8-aminoquinolines, and many other drugs, cause an acute intravascular haemolysis, known as primaquine-sensitivity, in a certain percentage of persons, particularly the darker-skinned peoples of the world. Massive drug programmes for the eradication of malaria in whole population groups frequently call for the use of primaquine; in addition, the use of other haemolytic or potentially haemolytic drugs in clinical medicine is widespread. Thus it is becoming increasingly important to be able to identify primaquine-sensitive individuals in field and clinical laboratories. Two modifications of a new test for primaquine-sensitivity, the methaemoglobin reduction test, are described in detail in this paper. The more simple modification, the field screening test, is practical for surveying large population groups in the field. The more accurate clinical test is also suitable for field use if a clinical spectrophotometer or photoelectric colorimeter is available.

INTRODUCTION

Primaquine-sensitivity (also called glucose-6-phosphate dehydrogenase deficiency) is an inborn error of metabolism. Individuals who inherit this defect experience an acute haemolytic episode after the ingestion of certain drugs and some vegetable foods, e.g., fava beans. The administration of 30 mg of primaquine base daily, twice the recommended antimalarial dose, (or toxicologically equivalent doses of various other drugs) to a person with full expression of this genetic defect may in some circumstances precipitate an acute intravascular haemolysis of such severity that it mimics blackwater fever. Many drugs can cause primaquine-type haemolysis, and the list of these drugs has expanded rapidly as clinical recognition of the entity has spread. It has recently been shown that when the trait is fully expressed (as in sensitive Negro males), the erythrocytic life-span is shortened even in the absence of drug administration (unpublished observations of Brewer, Tarlov, Kellermeyer & Alving).

The genetic defect occurs in approximately 10%-15% of American Negroes, and is common in other dark-skinned races in many areas throughout the world. It appears to be most prevalent in the Mediterranean peoples. Its incidence, in general, follows the same geographical pattern as the distribution of malaria.

The erythrocytes of primaquine-sensitive individuals have many intrinsic biochemical abnormalities. A decrease of glucose-6-phosphate dehydrogenase (G-6-PD) activity, first shown to be characteristic of primaquine-sensitivity by Carson, Flanagan, Ickes & Alving (1956), may represent the major enzymatic deficiency of the erythrocytes. The usual transmission of the trait appears to be by a partially dominant sex-linked (X-chromosome) gene; with rare exceptions affected males show full expression, and are presumably hemizygous; females usually exhibit intermediate expression and are presumably heterozygous. The occasional female with full expression is considered to be homozygous. The mechanism of inheritance may be variable; a sex-influenced autosomal gene has not been excluded in some familial studies.
TESTS FOR PRIMAQUINE-SENSITIVITY

All tests for primaquine-sensitivity, with the exception of the reduced glutathione (GSH) content of erythrocytes (Beutler, Dern, Flanagan & Alving, 1955), and the Heinz body test (Beutler, Dern & Alving, 1955), are adequate for differentiating males with full expression of the trait from those who are non-sensitive; but no currently available test differentiates with complete confidence intermediate females from those unaffected, on the one hand, and those exhibiting full expression, on the other. Some tests, moreover, call for special technical skill; others are costly or require chemicals and instruments not commonly available in clinical laboratories or the field. The two tests most widely used for screening large populations are: (1) the GSH stability test of Beutler, Robson & Buttenweiser (1957), and (2) the dye test devised by Motulsky, Kraut, Thieme & Musto (1959). Primaquine-sensitivity is determined in the GSH stability test by the in vitro destruction of GSH during incubation of erythrocytes with acetylphenylhydrazine. In the dye test, sensitivity is detected by the failure of haemolysates to change the colour of brilliant cresyl blue. Probably both tests are based on the inability of sensitive erythrocytes, deficient in G-6-PD, to generate reduced triphosphopyridine nucleotide (TPNH), the dye test directly, and the GSH stability less directly, because of its dependence on a second enzyme, glutathione reductase.

Two modifications of a new test, the “methaemoglobin reduction test” for primaquine-sensitivity, are described in this paper. Both predict more accurately than previous tests the effect of drug on the life-span of erythrocytes. The more simple “field screening test”, which is the most practical for surveys of large population groups, does not require optical instruments for interpretation of results: the reduction of methaemoglobin (MetHb) to haemoglobin (Hb) is determined by gross inspection. It is inexpensive and requires only equipment and reagents commonly available. The more accurate “clinical test” requires an optical instrument for interpretation of results and is preferable for use in blood banks and clinical laboratories. It is also suitable for use in the field if a photelectric colorimeter or clinical spectrophotometer is available. In these tests, primaquine-sensitivity is determined by the inability of erythrocytes to accelerate the reduction of MetHb when incubated with glucose, sodium nitrite and methylene blue.

MATERIALS AND METHODS

REAGENTS

Reagents required for both the field screening and the clinical tests

0.145 M sodium chloride solution (normal saline). Place 8.5 g of sodium chloride in a 1-litre volumetric flask, mix well and bring to volume with distilled water.

0.28 M glucose solution. Place 5.0 g of glucose in 100-ml volumetric flask, mix well and bring to volume with distilled water. This solution, if sterile, will keep indefinitely; if unsterile, it will usually keep for at least one week under refrigeration (4°-8°C). If unsterile and unrefrigerated, it should be freshly made each day.

Acid-citrate-dextrose (ACD) solution. Place 2.45 g of dextrose (glucose), 0.8 g of citric acid, and 2.2 g of trisodium citrate in a 100-ml volumetric flask, mix well and bring to volume with distilled water. The same storage precautions hold as for the glucose solution described in the preceding paragraph.

Acid-citrate-dextrose-inosine (ACDI) solution. Place 2.4 g of inosine in a 100-ml volumetric flask, mix well and bring to volume with ACD solution. The same storage precautions hold as for the 0.28 M glucose solution above. A white precipitate may form when this solution is refrigerated, but this will dissolve if the solution is gently warmed (37°C).

0.18 M sodium nitrite solution. Place 1.250 g of sodium nitrite in a 100-ml volumetric flask, mix well and bring to volume with distilled water. This solution should be kept in a tightly stoppered, light-sheltered bottle and should be freshly made each month. This solution is made from distilled water rather than from normal saline because it is slightly hypertonic with respect to blood.

1 Unless otherwise specified, reagent-grade chemicals are used throughout.
0.0004 M methylene blue chloride solution. Place 149.5 mg of trihydrated methylene blue chloride (Mallinkrodt; mol. wt. 373.92—preparation corresponding to National Formulary) in a 100-ml volumetric flask, mix well and bring to volume with distilled water. This solution may require gentle warming (37°C) to effect complete solution. Make a 1:10 dilution by placing 10 ml of this solution in a 100-ml volumetric flask and bring to volume with 0.145 M sodium chloride solution. Discard the remainder of the concentrated solution, which should not be held over for later use. The 1:10 dilution is the solution to be used and should be freshly made each month. The final dilution is made with normal saline rather than from distilled water in order to make the solution approximately isotonic with respect to blood.

0.02 M KH₂PO₄-NaOH buffer, pH 6.6 (0.02 M phosphate buffer). Place 3.4 g of KH₂PO₄ in a 125-ml volumetric flask, mix well and bring to volume with distilled water. Place this 125-ml solution in a 500-ml volumetric flask, and add 44.2 ml of 0.19 M sodium hydroxide, mix well and bring to volume with distilled water. This is a 0.05 M phosphate buffer with a pH of 6.6, and is used in the clinical test but not the field screening test. Place 400 ml of this solution in a 1-litre volumetric flask, mix well and bring to volume with distilled water. This is a 0.02 M phosphate buffer with pH 6.6. The pH of this buffer is usually quite close to 6.6 if these instructions are followed; however, since the pH is of importance, it should be checked initially and occasionally thereafter with the best means available, preferably some type of pH meter.

Additional reagents required for the determinations of MetHb and Hb in the clinical methaemoglobin reduction test

0.4 M sodium cyanide solution. Place 1.0 g of sodium cyanide in a 50-ml volumetric flask, mix well and bring to volume with distilled water. This solution should be kept in the refrigerator at 4°-8°C in a light-sheltered bottle and should be freshly made each month. If a refrigerator is unavailable, the solution should be freshly made each week. (Caution: It must be remembered that sodium cyanide is poisonous and care should be taken to avoid ingestion by mouth or excessive inhalation of the vapour. Crosby, Munn & Furth (1954) have discussed this topic with respect to the international use of cyanide for Hb determinations.)

0.6 M potassium ferricyanide solution. Place 10.0 g of potassium ferricyanide in a 50-ml volumetric flask, mix well and bring to volume with distilled water. This solution should be kept in the refrigerator at 4°-8°C in a light-sheltered bottle, and is stable for several months. If a refrigerator is unavailable, the solution should be freshly made each month.

0.05 M KH₂PO₄-NaOH buffer, pH 6.6 (0.05 M phosphate buffer). Place 13.6 g of KH₂PO₄ in a 500-ml volumetric flask, mix well and bring to volume with distilled water. Place this 500-ml solution in a 2-litre volumetric flask and add 177 ml of 0.19 M sodium hydroxide, mix well, and bring to volume with distilled water. This is a 0.05 M buffer with a pH of 6.6. The same precautions hold with respect to checking the pH as for the 0.02 M phosphate buffer described above.

METHAEMOGLOBIN REDUCTION TEST

Principle

The methaemoglobin reduction test for primaquine-sensitivity consists of the oxidation of Hb to MetHb by sodium nitrite and the subsequent enzymatic reconversion to Hb in the presence of methylene blue; this occurs by stimulation of the pentose phosphate pathway and activation of TPNH methaemoglobin reductase. Other ancillary enzymatic and non-enzymatic mechanisms for the reduction of MetHb may play a role.

General procedure for methaemoglobin reduction test (same for field screening and clinical tests)

Collection, preparation and storage of blood samples. Venous blood can be mixed with heparin (0.2 mg per ml of whole blood) or ACD (0.15 ml of ACD per ml of whole blood) if the test is to be performed within eight hours of obtaining the blood. If heparin is used as the anticoagulant, add 0.1 ml of 0.28 M glucose solution per ml of whole blood. If the test is not to be started within 30 minutes of the blood's being taken from the vein, the blood should be chilled (4°-8°C) until ready for use. If the test is not performed within eight hours of drawing the blood, weakly false positive results may be obtained unless ACDI (0.15 ml per ml of whole blood) is used as the anticoagulant. If the blood is refrigerated and stored with ACDI, it can be kept for at least one week and reliable results can still be obtained.
Oxidation of haemoglobin to methaemoglobin by sodium nitrite and subsequent enzymatic reconversion to haemoglobin in the presence of methylene blue. Place 2.0 ml of the blood sample in a test-tube (internal diameter between 10 mm and 15 mm, height between 10 cm and 15 cm). Add 0.1 ml of 0.18 M sodium nitrite solution. Then add 0.1 ml of 0.0004 M methylene blue chloride solution. Mix the sample by gentle inversion twelve times, and place it in a water-bath at 37°C (± 0.5°C). Place a 0.1-ml pipette in each tube for use in aeration and to remove the 0.1 ml of blood at the end of the incubation. At 60 and 120 minutes after the start of the incubation, mix the sample by stirring with the 0.1-ml pipette and blow one breath of air by mouth gently through the pipette into the blood; bubbles should not go over the top of the test-tube. At 180 minutes the incubation is finished.

Precautions for the general procedure. The following precautionary measures should be observed:

(a) Supplementary glucose. The reduction of MetHb is dependent upon an adequate supply of glucose, and false positive results will be obtained if glucose is not present in sufficient quantity. Therefore, supplementary glucose, ACD, or ACDI must be added to every blood sample, as described in the description of the collection, preparation and storage of blood samples.

(b) Storage of blood if test is not to be performed promptly. If blood is allowed to stand for a time at room temperature after it is drawn, false negative results will be obtained in some intermediate females. It should therefore be kept chilled (4°-8°C), as mentioned above, and then the start of the test can be delayed up to eight hours if the blood is in heparin and glucose or in ACD. If ACDI is used and the blood kept refrigerated at all times, it can be stored for at least seven days and reliable results can still be obtained.

(c) Aeration. The hourly aeration procedure is critical and should be followed carefully. The number of times air is introduced, as well as the amount of air, influences the results in intermediate females. Too frequent or too vigorous aeration may give weak false positive results, and no aeration may cause some intermediate individuals to show falsely negative results. In practice, we have found that by the simple expedient of blowing one breath of air through the blood at 60 and 120 minutes after the start of incubation, reproducible and reliable results are obtained. Because of the influence of oxygen saturation, we also recommend that the incubation test-tubes be within the size limits specified. We have tested tubes of these size ranges and have found that within these limits, results are not affected.

(d) Incubation temperatures. MetHb reduction is accelerated with increasing temperatures and is slowed with decreasing temperatures; therefore the incubation temperature should be carefully maintained between 36.5°C and 37.5°C. This requirement is easily met by most laboratory water-baths with thermostatic controls. In a later section, the use of a 43°C incubation temperature for hot, tropical areas is described.

Subsequent procedure for field screening test

Estimation of remaining MetHb. Pipette 0.1 ml of the incubated mixture into 10.0 ml of 0.02 M phosphate buffer, pH 6.6, in a test-tube (internal diameter between 10 mm and 30 mm). Treat the artificial standards similarly (see “Standards for field screening test” below). Between 2½ and 5 minutes after adding the 0.1 ml of blood, visually inspect these tubes and compare the unknown samples with the standards.

Interpretation of the results of field screening test. The results of the field screening test make it possible to group persons in one of the three following categories:

(a) Non-sensitive individuals. The colour of the diluted sample is clear red, like the normal standard.

(b) Intermediate expression (heterozygous females). The colour of the diluted sample varies in accordance with the degree of expression of the trait. The sample in strongly expressed intermediate females is dark grey or brown, like the positive standard. The samples of very weakly expressed females may be indistinguishable from the normal standard described above for non-sensitive individuals. (These individuals have only a very mild haemolysis with 30 mg of primaquine base daily). Other intermediate females vary in gradation between these two extremes.

(c) Full expression (hemizygous males, homozygous females). The colour of the diluted sample is dark grey or brown, like the positive standard.

Standards for field screening test. It is not necessary to run known normal and primaquine-sensitive blood as controls for visual comparison. Artificial standards can be produced from any convenient blood sample (primaquine-sensitive or normal) providing the haematocrit or Hb value is
INTERPRETATION OF RESULTS OF CLINICAL TEST

<table>
<thead>
<tr>
<th>Type of individual</th>
<th>Percentage of total Hb pigment remaining as MetHb at the end of incubation</th>
</tr>
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<tbody>
<tr>
<td>Non-sensitive</td>
<td>5 or less</td>
</tr>
<tr>
<td>Intermediate expression (heterozygous females)</td>
<td>5-80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Full expression (hemizygous males, homozygous females)</td>
<td>80-95</td>
</tr>
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<sup>a</sup> The percentage of pigment remaining as MetHb varies between 5 and 80 in accordance with the degree of expression of the trait in intermediate females. Those females with test values near the 80% extreme are strongly expressed and those near the 5% extreme are weakly expressed. These values correlate well with the severity of haemolysis when a standard challenge course of primaquine is administered.

Modification of methaemoglobin reduction test for areas where a 37°C incubation temperature is not feasible owing to high environmental temperatures

In tropical or other areas where the test must be conducted in an environmental temperature which exceeds 37°C, the test may be performed at 43°C in order to make possible the use of a standard laboratory incubator. When the temperature in increased, the rate of reduction of MetHb is accelerated and the incubation time must therefore be shortened. The test results obtained at 43°C agree closely with those found at 37°C, if an incubation time of 150 minutes rather than 180 minutes is used. At temperatures higher than 43°C test results become variable. When the test is being performed at 43°C the procedure and interpretation of results are exactly the same in both the field screening test and the clinical tests, as described in the previous sections, except for the shortened incubation period.

METHAEMOGLOBIN AND HAEMOGLOBIN DETERMINATIONS

(Necessary for the clinical test only)

**Principle**

Methaemoglobin and haemoglobin are determined by a modification of the Evelyn & Malloy (1938) method, adapted to the Coleman Junior Spectrophotometer. The determination of MetHb is dependent upon the change of optical density at 640 µ wavelength when sodium cyanide is added to a solution containing MetHb, which converts...
the MetHb to cyanmethaemoglobin. Haemoglobin is determined on the same sample by determining the optical density of the solution at 540 m\(\mu\) wavelength after converting all pigment to cyanmethaemoglobin with potassium ferricyanide and sodium cyanide.

**Procedure**

**Cuvettes.** We use Coleman Junior Spectrophotometer cuvettes, 19 mm outside diameter, class A or B.

**Delivery of blood sample.** Pipette 0.1 ml of the incubated blood sample with a 0.1-ml pipette into 10.0 ml of 0.02 M phosphate buffer, pH 6.6, in a cuvette and stir the solution with the pipette.

**Reading 1.** Allow the cuvette to stand for at least 2½ minutes in order to obtain complete lysis of erythrocytes and minimal turbidity. Meanwhile, use a 0.02 M phosphate buffer blank to adjust the optical density of the Coleman Junior Spectrophotometer to zero. Between 2½ and 5 minutes (see the precaution regarding timing below) after addition of the 0.1 ml of incubated sample, determine the optical density at 640 m\(\mu\) wavelength.

**Reading 2.** Place one drop (dropper calibrated to deliver 16-20 drops per ml) of 0.4 M sodium cyanide in the blank cuvette and in the sample cuvette, and stir the solution with a stirring rod. After 5 minutes or more, determine the optical density at 640 m\(\mu\) wavelength in the manner described for Reading 1.

**Calculating g % MetHb.** Reading 1 minus Reading 2 in optical density units is directly proportional to the amount of MetHb originally present. Convert this value to g % MetHb by multiplying with the appropriate factor. (It is advisable to determine this factor for each instrument; see “Standardization of MetHb method” below).

**Determination of total Hb pigment (Hb plus MetHb).** Determine the total Hb on the same sample by converting all pigment to cyanmethaemoglobin in the following manner:

(a) Add 2 ml of the solution in the Coleman cuvette used for Reading 2 to 8.0 ml of 0.05 M phosphate buffer in a second Coleman cuvette.

(b) Add one drop of 0.6 M potassium ferricyanide and one drop of 0.4 M sodium cyanide to the second cuvette and mix the solution with a stirring rod. This procedure converts all pigment to cyanmethaemoglobin. Allow this solution to stand for at least 10 minutes. Prepare a blank by pipetting 2.0 ml from the blank used for Reading 2 into 8.0 ml of 0.05 M phosphate buffer, and then add one drop of 0.6 M potassium ferricyanide and one drop of 0.4 M sodium cyanide. Use this blank to adjust the optical density of the instrument to zero, and determine the optical density of the sample in the second cuvette at 540 m\(\mu\), being sure that 10 minutes have elapsed after the 2 ml were added. This is Reading 3. Convert this value in optical density units to g % Hb by multiplying with the appropriate factor. (It is advisable to determine this factor for each instrument; see “Standardization of Hb method” below.)

**Precautions for MetHb and Hb determinations**

In determining Reading 1, the optical density should be obtained between 2½ and 5 minutes after the 0.1 ml of incubation mixture is pipetted into the 10.0 ml of 0.02 M phosphate buffer. The reasons for this are that it takes slightly more than 2 minutes for the erythrocytes to be lysed and for turbidity to become minimal; on the other hand, after the erythrocytes are lysed they are no longer able to reduce MetHb, and Hb is converted to MetHb in the cuvette because of the continued presence of sodium nitrite. This takes place slowly in normal blood, but rapidly in blood from anaemic individuals. The error introduced because of these factors is minimal if Reading 1 is determined between 2½ and 5 minutes after the addition of the 0.1 ml of incubated sample.

**Standardization of Hb method**

The Hb method may be calibrated with several dilutions of Hemotrol (Clinton Laboratories), a Hb standard. Treat 0.1 ml of each of the Hemotrol dilutions in exactly the same manner as if it were a blood sample, and follow the procedure discussed above under the heading “Methaemoglobin and haemoglobin determinations”, except that only Reading 3 need be determined. In this way, the optical density of known Hb solutions can be determined with the particular instrument in use, and a standard curve constructed.

**Standardization of MetHb method**

Place 1 ml of any sample of whole blood in a 100-ml volumetric flask and bring to volume with the 0.02 M phosphate buffer, pH 6.6. Place this haemolsate in a 250-ml Erlenmeyer flask and add 6.24 mg of potassium ferricyanide. This procedure changes all of the Hb to MetHb, and is used for
standardization in preference to sodium nitrite because of Austin & Drabkin's (1935) recommendations. Make several dilutions from the haemolysate, and then use 10 ml of the various dilutions to obtain Reading 1 and Reading 2 as described above. Use 2 ml of the solution for Reading 2 to obtain Reading 3, as also described above. Translate Reading 3 into g% Hb using the factor derived from the Hemotrol standardization. The g% Hb of each dilution is equal to the g% MetHb originally present, since 100% of the pigment was in the form of MetHb. Construct a standard curve relating Reading 1 minus Reading 2 in optical density units to g% MetHb.

**DISCUSSION**

Energy for the specific enzymatic reduction of MetHb is derived from glucose metabolism in the mature human erythrocyte. At least two routes are known to be involved: (1) the glycolytic pathway, through which approximately 90% of glucose is normally channelled (Murphy, 1960), and which supplies energy for reduced diphosphopyridine nucleotide (DPNH) MetHb reductase; and (2) the pentose phosphate pathway, which yields energy for TPNH MetHb reductase. Under physiological conditions, the conversion of MetHb to Hb seems to be almost entirely catalysed by DPNH MetHb reductase (Gibson, 1948). However, when the activity of the pentose phosphate pathway is markedly accelerated by an artificial electron carrier, such as methylene blue, the rate of reduction of MetHb by TPNH MetHb reductase is greatly increased (Gibson, 1948). In contrast, the rate of reduction of MetHb by DPNH MetHb reductase is increased only slightly by methylene blue (Gibson, 1948; Jaffe, 1959). The accompanying figure demonstrates the two paths of carbohydrate metabolism in erythrocytes and the routes of specific enzymatic MetHb reduction. The decreased activity of G-6-PD in primaquine-sensitive erythrocytes results in diminished generation of TPNH from TPN and, as a consequence, leads to defective reduction of MetHb by TPNH MetHb reductase in the presence of methylene blue (Dawson, Thayer & Desforges, 1958; Ross & Desforges, 1959; Lohr & Waller, 1958). The methaemoglobin reduction test for primaquine-sensitivity probably reflects the absolute or relative inability of sensitive erythrocytes to generate TPNH for MetHb reduction. Other secondary mechanisms of MetHb reduction have not been excluded as factors in the test.

Most primaquine-sensitive males are fully susceptible to drug-induced haemolysis and presently used tests can distinguish them from normals successfully. Most affected females, however, have an intermediate susceptibility to haemolysis, have extremely variable biochemical changes in their erythrocytes, and are difficult, if not impossible, to distinguish by currently available tests. The methae-
The glucose-6-phosphate dehydrogenase activity with the severity of \textit{in vivo} haemolysis in male and female volunteers in whom haemolysis was determined following the ingestion of a standard challenge dose of primaquine (30 mg base daily, administered for a period of 14 days).

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**REFERENCES**

Gibson, Q. (1948) \textit{Biochem. J.}, \textbf{42}, 13
Jaffe, E. (1959) \textit{J. clin. Invest.}, \textbf{38}, 1553
Murphy, J. (1960) \textit{J. Lab. clin. Med.}, \textbf{55}, 286
Ross, J. & Desforges, J. (1959) \textit{J. Lab. clin. med.}, \textbf{54}, 450