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SICKEL-CELL DIAGNOSTIC TEST

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ABSTRACT OF THE DISCLOSURE

A diagnostic test and reagent for the rapid identification of sickle-cell anemia and sickle-cell trait comprising reaction of unclotted blood with an admixture of $(NH_4)_2SO_4/K_2HPO_4$ buffer, pH 7–7.5, saponin and sodium dithionite and observation of the turbidometric-colormetric characteristics.

This invention relates to a diagnostic test and reagent. More particularly, this invention relates to a method for the rapid identification of sickle-cell anemia and sickle-cell trait.

The term sickle-cell disease refers to conditions and diseases characterized by the presence of hemoglobin S (HbS). Hemoglobin S is an inherited characteristic and is probably the most widely encountered hemoglobin variant in the United States. When present in the individual, hemoglobin S is either homozygous (SS—sickle-cell anemia) or heterozygous (AS—sickle-cell trait). It can also be found in combination with other abnormal hemoglobins and with other hereditary diseases. The importance of adequate diagnosis of sickle-cell disease lies in the fact that if unrecognized and/or untreated, the disease may be fatal. Under certain conditions that cause low oxygen tension, sickle-cell trait can result in serious and even fatal clinical complications. Sickle-cell anemia may prove fatal before adolescence but with appropriate medical care it is possible to extend survival for 30 or 40 years.

Various methods of diagnosis of sickle-cell disease have been reported heretofore. They include electrophoresis, differential solubility tests and slide elution tests. Of these, the original differential solubility test of Itano, J. of Haematology, 4, pp. 66–68 (1949), Arch. Biochem. Biophys, 47, pp. 148–59 (1953), Science, 117, pp. 89–94 (1953), and modifications thereof are most prevalent in use. One such modification described in U.S. Pat. 3,492,-095 involves the steps of adding sodium dithionite reductant to a high ionic concentration phosphate buffer system, adding a saponin hemolyzing agent thereto and then adding a given amount of the blood to be tested. After mixing and standing for a period of time, the resultant solution is observed for translucense or turbidity. Turbidity indicates the presence of hemoglobin S whereas if the solution remains translucent, the results indicate the absence of hemoglobin S.

While the foregoing modification is useful for determining the absence or the probable presence of hemoglobin S, it does not provide for differentiation between sickle-cell anemia (SS) and sickle-cell trait (AS).

Accordingly, it is an object of this invention to provide a diagnostic test and reagent for the determination of hemoglobin S.

It is another object of this invention to provide a diagnostic test and reagent for the rapid identification of sickle-cell anemia and sickle-cell trait.

Other objects and advantages of the present invention will be apparent to those skilled in the art after reading the disclosure hereof.

In brief, the present invention resides in the provision of a new buffer system and a macroscopic turbidometric-colorimetric procedure for distinguishing between sickle-cell anemia and sickle-cell trait.

According to past practice it has been customary to use a phosphate buffer of high ionic concentration. Thus, U.S. Pat. 3,492,095 describes the use of a buffer containing 16.9 grams of KH_2PO_4 and 21.7 grams of K_2HPO_4 (or a total of 38.6 grams of phosphate salts) in 100 ml. of water and having a high hydrogen ion concentration and concomitant pH of 6.5 to 6.8. In the present invention the new buffer system comprises essentially $(NH_4)_2SO_4$ of relatively low ionic concentration and only a small amount of K_2HPO_4. The $(NH_4)_2SO_4$, which at normal room temperature (20–25° C.) forms a 76% saturated solution in water, is used at about one-third saturation and preferably at a level of from about 21 to about 35 grams per 100 ml. Admixed with the $(NH_4)_2SO_4$ to form the present buffer is about 0.5 to about 2 grams of K_2HPO_4 per 100 ml. Thus, the overall buffer solution has a relatively low ionic concentration in terms of the saturation point and only a small fraction thereof comprises phosphate. Instead of having a high hydrogen ion concentration, a relatively low hydrogen ion concentration is employed in the buffer of the present invention whereby the pH is neutral to slightly alkaline and preferably from about 7 to about 7.5. For example, a representative buffer of the present invention having a pH of 7.1 has only one-fourth (¼) the number of hydrogen ions that are in a buffer having a pH of 6.5.

In addition to the new buffer system described herein, the reagents employed in the present invention include a red cell hemolytic agent such as, for example, saponin and a reducing agent such as, for example, sodium dithionite. The use of saponin as a rapid hemolytic agent for measuring hemoglobin content has been disclosed heretofore in U.S. Pat. 2,519,999, col. 51, lines 2–10, and the use of sodium dithionite as a reducing agent in a test for hemoglobin has been described previously by Itano and Pauling, J. of Haematology, 4, pp. 66–68 (1949). However, it has not been known heretofore to use the latter two reagents in combination with the new buffer system defined herein. By employing these three reagents together it has now been made possible to obtain a convenient and rapid identification of sickle-cell anemia and sickle-cell trait in one test whereas previously it was possible only to obtain identification of hemoglobin S with the high ionic concentration phosphate buffer, saponin, and sodium dithionite. In said prior test it had been necessary to conduct a separate and cumbersome electrophoresis test to differentiate between sickle-cell anemia and sickle-cell trait, thereby disadvantageously requiring a second visit to the clinical laboratory by all those patients whose initial test was found to be positive for hemoglobin S. Health Services and Mental Health Administration Health Reports, 87 (1), pp. 9–12 (1972).

In accordance with the method of this invention, the hemolytic agent is mixed with the buffer and then the reductant is admixed therewith. A sample of test blood such as, for example, from a finger or heel puncture or anticoagulated venous blood is then mixed with a suitable aliquot of the resultant reagent mixture in a micro test tube and the solution allowed to stand at normal room temperature for several minutes, e.g., about five minutes, after which the test tube is viewed in the path of a fluorescent light with a metal reflector to determine whether the solution is translucent or turbid. If the solution is transulcent, the hemoglobin is AA (normal), whereas if the solution is tubid, then the hemoglobin is AS (sickle-cell trait) or SS (sickle-cell anemia).

A further differential of the gene types can then be made in the same test by spinning the test tube with the turbid sample in a serological centrifuge for several minutes, e.g., about three minutes, at about 3000 to 5000

r.p.m. At the end of this time period the test tube is viewed again for color characteristics in the fluorescent light path.

Hemoglobin S is insoluble in the new buffer system of this invention and the SS hemoglobin is a yellow solution with red precipitate. The AS hemoglobin is a pink solution with a red precipitate. Normal AA hemoglobin is soluble in the new buffer system of this invention with only a very small amount of a white precipitate.

The method of this invention has been found to give a 100% correlation between the S gene and hemoglobin electrophoresis. It should be understood, however, that the method does not differentiate the beta thalassemia (Th) or C gene and there is a potential error or two persons out of 100 being diagnosed as AS when they could be CS or ThS. There is a potential error of 3.8% in diagnosis as AA when the patient may have an additional hemoglobin problem of Th or a C gene.

It is thus seen that the diagnostic test and reagent of the present invention provides for the rapid identification of sickle-cell anemia and sickle-cell trait in one test with only a small recognizable margin of error in the diagnosis of other hemoglobinopathies.

A further advantage of the present invention is that the differentiation of sickle-cell anemia and sickle-cell trait is carried out by observation of a sharply defined precipitate which rapidly settles at the bottom of the test tube and a distinct color difference in the supernantant solution. Differentiation between sickle-cell trait and sickle-cell anemia has been disclosed heretofore by Huntsman et al., J. Clin. Path., 23, pp. 781–83 (1970), but in the method described therein the turbid material rises to the surface of the solution. This causes significant problems due to the inability to obtain complete separation, the necessity of spinning in a hematocrit centrifuge, and the tendency of the entire precipitate to drop from the surface during the centrfuging or braking of the centrifuge and in the normal handling of the test tubes in the laboratory thereafter. This fragility of the turbid material at the top of the test tube whereby it falls into the solution tends to give false readings of the sickle-cell diagnosis.

The transillumination of the micro test tube containing the sickle-cell diagnostic reagent and the test blood sample can be conveniently carried out in a fluorescent illuminator such as described in the co-pending utility application of Seitz and Miranda, Ser. No. 237,824, filed concurrently herewith and assigned to a common assignee. A micro test tube, 12 mm. diameter x 75 mm. long, can have a dark letter A, about 5–10 mm. high, printed on the side of the tube opposite the viewer but adjacent the illuminator. So long as the solution remains translucent, the letter A can be read through the tube, but when the solution becomes turbid, the letter A can no longer be seen through the tube.

In accordance with a preferred embodiment of the invention, the new buffer system is provided together with the other reagents in a complete kit form for the diagnostic test. The buffer can be in liquid form but it is preferred to provide the hemolytic agent and reductant in a dry, powdered form to extend the shelf life of the test kit. In particular, the reductant sodium dithionate tends to lose SO_2 in aqueous solution over a period of time.

Prior to use, the powdered hemolytic agent and the powdered reductant or an admixture thereof can be admixed with the buffer solution and the resulting solution can then be used to conduct a plurality of diagnostic tests for sickle-cell disease. Thus, 0.55 grams each of saponin and sodium dithionite in 55 ml. of buffer solution is sufficient for about 25 sickle-cell diagnostic tests.

According to yet another embodiment of the invention, the buffer solution contains a re-dox indicator dye such as, for example, methylene blue, which will change color by virtue of the break-down of the sodium dithionite reductant during storage when admixed with the aqueous buffer solution. Thus, methylene blue is colorless in the reduced state and blue in the oxidized state. So long as the buffer containing the sodium dithionite remains colorless, or yellow due to the pressure of the saponin hemolytic agent, the reagent system is suitable for carrying out the diagnostic test of this invention. At such time as the buffer system turns blue due to the break-down of the sodium dithionite during storage and conversion of the methylene blue to the oxidized state, the reagent should be discarded.

Another suitable re-dox indicator dye system which can be used in this invention is basic fuchsin, which is violet red in oxidized form and colorless in reduced form.

The diagnostic test and reagent of this invention also lends, itself well for use on automated analytical equipment. For example, it can be adapted for application on the Autoanalyzer AAII instrument manufactured by Technicon Corp. and on various single channel instruments.

The following examples will further illustrate the present invention although it will be understood that the invention is not limited to these specific examples.

EXAMPLE 1

A complete sickle-cell diagnostic kit is provided with one hundred (12 mm. x 75 mm.) micro test tubes, each being calibrated at 2 ml. and having a dark letter A, 6 mm. high, printed on the outside; two bottles, each containing 105 ml. of a special buffer; two bottles, each containing 1.1 grams of dry, powdered saponin; and two bottles, each containing 1.1 grams of dry, powdered sodium dithionite. This kit is adapted for carrying out 100 sickle-cell diagnostic tests.

The special buffer is prepared as follows: a 28% solution of $(NH_4)_2SO_4$ is made-up by dissolving 280 grams of the sulfate salt in one liter of distilled water. A molar solution of K_2HPO_4 is made-up by dissolving 174 grams of the phosphate salt in one liter of distilled water. To one liter of the $(NH_4)_2SO_4$ solution is then added 60 ml. of the K_2HPO_4 solution. The pH of the resulting buffer is 7.1.

One unit of 1.1 gram of saponin is mixed with one unit of 105 ml. of buffer solution and then one unit of 1.1 gram of sodium dithionate is admixed therewith. The resulting solution is stable for about 30 days at about 2°–8° C. and is suitable for carrying out the diagnostic method of this invention for the rapid identification of sickle-cell anemia and sickle-cell trait.

EXAMPLE 2

Diagnosis for sickle-cell disease by use of the final reagent solution for Example 1, above, is carried out as follows:

An aliquot comprising 2 ml. of the reagent solution is pipetted into one of the micro test tubes. A one-tenth (1/10) ml. sample of the patients' blood, which is non-clotted, is then mixed into the solution and the mixture allowed to stand at room temperature for five minutes. After the five minute setting period, the test tube and reagen solution is viewed in a fluorescent illuminator to determine whether the solution remains translucent (HbA) or becomes turbid (HbS).

The test tube and reagent solution is then spun in a serological centrifuge for three minutes at 3500 r.p.m. and again viewed in the fluorescent illuminator to determine whether the solution is translucent (HbAA), or pink with a red precipitate (HbAS), or yellow with a red precipitate (HbSS).

The above differentiation between sickle-cell anemia (HbSS) and sickle-cell trait (HbAS) is 97% reliable with respect to all hemoglobinopathies and the determination of the sickle-cell gene (S) is 100% reliable. The entire test is conducted in only about 8 minutes, thereby providing a rapid diagnosis of the patient.

Although specific amounts and proportions of hemolytic agent and reductant are described in the foregoing examples, it will be apparent that variations from these examples can be made in the diagnostic test and reagent of

this invention without departing from the basic and novel concepts thereof. In general, these materials are each used within ranges of from about 0.01 to about 2 grams per 100 ml. of solution. Conventional hemolytic agents and reductants other than those specifically described herein can also be used with substantially equivalent results.

Various other examples and modifications of the foregoing examples will be apparent to the person skilled in the art after reading the above disclosure and the appended claims without departing from the spirit and scope of the invention. All such further examples and modifications are included within the scope of said claims.

What is claimed is:

1. A reagent for the diagnosis of sickle-cell disease comprising a mixture of an aqueous buffer solution, a hemolyzing agent and a reducing agent, said buffer comprising from about 21 to about 35 grams of $(NH_4)_2SO_4$ per 100 ml. and from about 0.5 to about 2.0 grams of K_2HPO_4 per 100 ml. and having a pH of from about 7 to about 7.5.

2. The reagent of claim **1** in which the buffer contains about 28 grams of $(NH_4)_2SO_4$ per 100 ml. and about one gram of K_2HPO_4 per 100 ml.

3. The reagent of claim **1** in which the hemolyzing agent is saponin.

4. The reagent of claim **1** in which the reducing agent is sodium dithionite.

5. The reagent of claim **1** in which the mixture contains about 28 grams of $(NH_4)_2SO_4$, about one gram of K_2HPO_4, about one gram of saponin, and about one gram of sodium dithionate per 100 ml. of solution.

6. A method for the rapid differentiation of sickle-cell anemia and sickle-cell trait comprising admixing a sample of unclotted blood with a predetermined amount of the reagent of claim **1** and observing macroscopically for turbidity in the solution as indicative of presence of HbS, then centrifuging and observing macroscopically for color characteristics in the solution in which a pink solution is indicative of sickle-cell trait and a yellow solution is indicative of sickle-cell anemia.

7. The method of claim **6** in which blood sample and reagent are admixed in proportions of about 100 microliters of blood and about 2 milliliters of reagent.

References Cited

UNITED STATES PATENTS

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