

[54] **DIAGNOSTIC TEST FOR SICKLE-CELL**

3,607,695 9/1971 Schneider..... 23/230 B

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[57] **ABSTRACT**

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A microcapillary method for the diagnosis of sickle-cell disease in which a sample of unclotted blood is mixed with a reagent comprising phosphate buffer containing a non-ionic surfactant having high water solubility, a hemolyzing agent and a reducing agent, and the reaction results observed macroscopically for turbidity and then a microcapillary sample thereof observed macroscopically for color characteristics.

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[51] Int. Cl. **G01n 21/06, G01n 33/16**

[58] Field of Search **23/230 B; 252/408**

[56] **References Cited**

UNITED STATES PATENTS

3,492,095 1/1970 Tillem 23/230 B

8 Claims, No Drawings

DIAGNOSTIC TEST FOR SICKLE-CELL

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. No. 3,492,095 involves the steps of adding sodium dithionate reductant to a high ionic concentration phosphate buffer system, adding a saponin hemolyzing agent thereto and then adding a given amount of blood to be tested. After mixing and standing for a period of time, the resultant solution is observed for translucence 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 possible presence of hemoglobin S, it does not provide for differentiation between sickle-cell anemia (SS) and sickle-cell trait (AS).

A further improvement on the original Itano differential solubility test for hemoglobin S has been reported by Huntsman et al., J. clin. Path., 23, pp. 781-83 (1970). This modification is reported to be able to differentiate sickle-cell anemia and sickle-cell trait. The Huntsman procedure employs the same general reagents as described in U.S. Pat. No. 3,492,095 but after admixing the blood sample with these reagents in a round-bottomed Kahn tube (77 x 13 mm), the tube is centrifuged at 2,500 to 3,000 rpm for 5 minutes. The centrifuge must not be braked and, consequently, another 5 minutes must be provided to allow the spinning to gradually slow down to a stop. The results of the test are observed after the cessation of the spinning and are as follows:

A clear or opalescent red solution with a variable amount of greyish protein on the surface indicates normal AA hemoglobin or the absence of hemoglobin S. In the case of sickle-cell trait, the solution of reduced hemoglobin will be clear and pink. The sickle-cell hemoglobin separates to the surface as a dark red band. With sickle-cell anemia, the solution will be clear and

straw colored with all the hemoglobin being found as a dark red band at the surface.

Although the Huntsman procedure offers the advantage of differentiating sickle-cell trait and sickle-cell anemia, it does not work well in practice due to the fragility of the precipitate at the top of the tube. Because of this fragility, the centrifuge must not be braked and the resulting time for conducting the test is thereby nearly doubled. But even when the centrifuge is not braked, the entire precipitate tends to drop from the surface during the centrifuging and in the normal handling of the test tubes in the laboratory thereafter. This fragility of the precipitate at the top of the test tube whereby it falls into the solution tends to give false readings of the sickle-cell diagnosis.

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 novel microcapillary method with a macroscopic turbidometric-colorimetric procedure for distinguishing between sickle-cell anemia and sickle-cell trait.

In this microcapillary method a special surfactant is added the sickle-cell test reagent system and, after admixing the sample of test blood therewith, the mixture is drawn up into a microcapillary tube and spun in a hematocrit centrifuge for about 1 to 3 minutes. After completion of the centrifuging, the results of the test are observed by a turbidometric-colorimetric procedure. In the latter procedure, an all pink solution indicates normal AA hemoglobin, a pink solution with a dark red clump at the top of the solution indicates the AS hemoglobin, and a clear or yellow solution with a red clump at the surface indicates the SS hemoglobin.

The new microcapillary method of the present invention differentiates sickle-cell anemia and sickle-cell trait without the inherent disadvantages of the Huntsman procedure. Moreover, it provides the positive advantages of reducing centrifugation time, giving a sharper delineation of the supernatant hemoglobin-stroma, overcoming faulty laboratory technique which results from braking the centrifuge or careless handling of the tubes and thereby avoiding false readings due to fragility of the precipitate at the surface.

The sickle-cell reagent system employed in this invention comprises a phosphate buffer, a red cell hemolytic agent and a reducing agent together with a special surfactant. The surfactant employed in this reagent system is a non-ionic detergent having high water solubility. A preferred water soluble non-ionic detergent is octyl phenoxy polyethoxy ethanol. Detergents of the latter type provide maximum water solubility and are available commercially from Rohm & Haas Co. under the trade-mark "Triton X," for example, "Triton X-100," "Triton X-165," "Triton X-205," "Triton X-305," and "Triton X-405."

Another suitable water soluble non-ionic surfactant is nonyl phenoxy polyethoxy ethanol which is available commercially under the trade-mark "Triton N-100."

Detergents of the foregoing types can be described by the general formula $RC_6H_4(OC_2H_4)_nOH$ wherein R is octyl or nonyl and n is at least three.

On the other hand, non-ionic surfactants having high lipid solubility such as the polyoxyethylene sorbitan fatty acid esters are not desired for use in the present invention.

The non-ionic surfactant can be employed in the sickle-cell reagent system in an amount ranging from about 0.1 to about 1 ml. per liter of solution and preferably at a level of about 0.4 ml. per liter.

The phosphate buffer of the reagent system can be a mixed potassium dihydrogen phosphate/di-potassium hydrogen phosphate buffer of from about 2 to about 2.5 molarity, the hemolytic agent can be saponin and the reducing agent can be sodium dithionite as described, for example, by Huntsman.

In practicing the present invention, the hemolytic agent is mixed with the buffer and then the reductant is admixed therewith. A sample of the 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 5 minutes, after which the test tube is viewed macroscopically and preferably in the path of a fluorescent light with a metal reflector to determine whether the solution is translucent or turbid. If the solution is translucent, the hemoglobin is AA (normal), whereas if the solution is turbid, then the hemoglobin is AS (sickle-cell trait) or SS (sickle-cell anemia).

A further differentiation of the gene types can then be made with the same reagent/test sample by withdrawing a microcapillary sample of the mixture, e.g., about 40 to 50 microliters, and spinning in a hematocrit centrifuge for about 1 to 3 minutes at about 10,000 rpm. Use of a Clay-Adams centrifuge such as described in U.S. Pat. No. 2,739,759, at 10,500 rpm for 3 minutes is a suitable example of the required centrifuging. At the end of this time period the micro sample is viewed again macroscopically for color characteristics as hereinbefore stated for differentiating sickle-cell anemia and sickle-cell trait. In the microcapillary method of this invention with the special surfactant in the reagent system the centrifuge can be braked without causing dropping or settling of the turbid precipitate into the solution. The tubes containing the samples also can be handled by the laboratory technician without any special care being taken due to fragility of the precipitate such as required heretofore.

With the diagnostic test and reagent of this invention it is also no longer necessary to conduct a separate and cumbersome electrophoresis test to differentiate between sickle-cell anemia and sickle-cell trait. The method of this invention gives a 100 percent 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 of two persons out of 1,000 being diagnosed AS when they could be CS or ThS. There is a potential error of 3.8 percent 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 with a

single blood sample of the patient and with only a small recognizable margin of error in the diagnosis of other hemoglobinopathies.

In accordance with a preferred embodiment of the invention, the reagents and other components of the diagnostic test are provided together in a kit form for the complete sickle-cell diagnosis. 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 dithionite tends to lose SO_2 in aqueous solution over a period of time. The special non-ionic surfactant is preferably employed directly in the buffer solution.

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. The buffer solution can be, for example, a 2.25 molar phosphate buffer, pH 7, containing about 0.4 ml. "Triton X-405" per liter. A solution of 33.78 grams of anhydrous potassium dihydrogen phosphate and 59.33 grams of di-potassium hydrogen phosphate in 250 ml. of distilled water will provide a suitable such buffer.

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 100 (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 buffer solution with a special surfactant; two bottles, each containing 1.1 grams of dry, powdered saponin; two bottles, each containing 1.1 grams of dry powdered sodium dithionite; 100 heparinized microcapillary tubes; and a pad of "Critoseal," which is a commercially available pliable clay sealant material for closing one end of the capillary tube after withdrawing the reagent/test sample and prior to centrifuging.

The buffer solution with the special surfactant is prepared as follows: A solution is prepared by dissolving 135.12 grams of KH_2PO_4 and 237.333 grams of K_2HPO_4 in 700 ml. of distilled water and making up to one liter. Then 0.4 ml. of "Triton X-405" is admixed therewith, the pH is adjusted to 7.1 ± 0.1 and the resulting solution is filtered through a "Millipore" filter using 0.45 μ and 0.22 μ pads to remove opaque material.

One unit of 1.1 grams of saponin is mixed with one unit of 105 ml. of the buffer solution and then one unit of 1.1 grams of sodium dithionite 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 of 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 1/10 ml. sample of the patient's blood, which is non-clotted, is then mixed into the solution and the mixture allowed to stand at room temperature for 5 minutes. After the 5 minute setting period, the test tube and reagent 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 with the blood of each positive (HbS) reaction is re-mixed and a microcapillary sample thereof (40 to 50 microliters) is withdrawn and the capillary tube sealed at one end with a sealant or by flaming. The microcapillary sample is then spun in a microhematocrit centrifuge at 10,500 rpm for 3 minutes and again viewed in the fluorescent illuminator to determine whether the solution is pink with a dark red clump at the top (HbAS) or yellow with a red clump at the top (HbSS).

The above differentiation between sickle-cell anemia (HbSS) and sickle-cell trait (HbAS) is 97 percent reliable with respect to all hemoglobinopathies and the determination of the sickle-cell gene (S) is 100 percent reliable. The entire test is conducted in 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 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 phosphate buffer solution having a molarity of from about 2 to about 2.5 and a pH of from about 7 to about 7.2, a hemolyzing agent and a reducing agent, said buffer solution additionally containing from about 0.1 to about one mil. per liter of a non-ionic surfactant having a high water solubility and being selected from the group consisting of substances having the general formula $RC_nH_{2n+1}(OC_2H_4)_nOH$ wherein R is octyl or nonyl and n is at least 3.

2. The reagent of claim 1 in which the non-ionic surfactant is octyl phenoxy polyethoxy ethanol.

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 dithionate.

5. The reagent of claim 1 in which the phosphate buffer is about 2.25 molar $KHPO_4/K_2HPO_4$.

6. The reagent of claim 1 in which the mixture contains about 135 grams of KH_2PO_4 , about 237 grams of K_2HPO_4 , and about 0.4 ml. of non-ionic surfactant per liter.

7. 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 withdrawing a microcapillary sample of the turbid solution and centrifuging in a hematocrit centrifuge and observing macroscopically for color characteristics in the solution in which a pink solution with a dark red clump at the surface is indicative of sickle-cell trait and a yellow solution with a red clump at the surface is indicative of sickle-cell anemia.

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

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