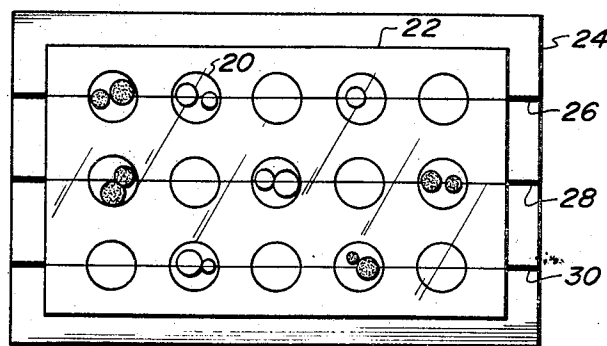
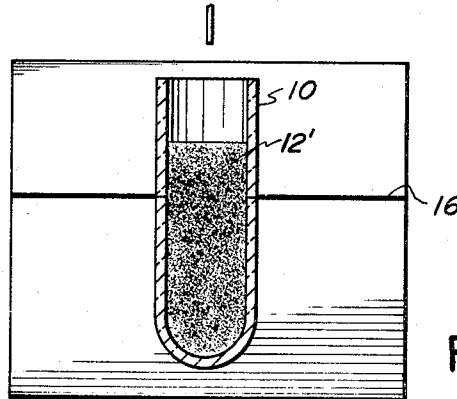
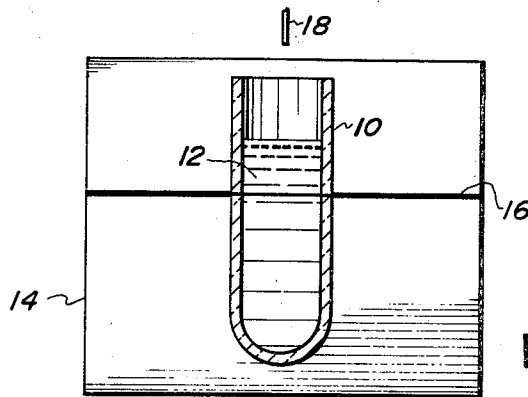


Jan. 27, 1970

H. B. TILLEM  
PRODUCT FOR AND METHOD OF TESTING BLOOD FOR  
THE PRESENCE OF HEMOGLOBINS  
Filed Oct. 5, 1967

3,492,095



INVENTOR.  
HAROLD B TILLEM  
BY *Lawrence J. Lerner*  
ATTORNEY

1

3,492,095

## PRODUCT FOR AND METHOD OF TESTING BLOOD FOR THE PRESENCE OF HEMOGLOBIN S

Harold B. Tillen, 17 Lowell Ave.,  
West Orange, N.J. 07052

Filed Oct. 5, 1967, Ser. No. 673,071

Int. Cl. G01n 31/02, 33/16

U.S. Cl. 23—230

19 Claims

### ABSTRACT OF THE DISCLOSURE

A method of and product for testing for the presence of Hemoglobin S comprising the steps of preparing a buffer solution, preferably a phosphate buffer and adding to the phosphate buffer a reducing agent such as sodium hydrosulfite. After dissolving the reducing agent in the buffer solution an erythrocytic hemolysis agent, such as a 2 percent saponin solution is added and mixed with the buffer and reducing agent. The resultant solution is then placed in a test tube and a measured amount of whole blood is added. The test tube contents are mixed and within two minutes, the results can be determined. That is, if the resultant solution is cloudy or turbid and shows a precipitate, Hemoglobin S is present. If the solution is opalescent or translucent, with no precipitate, then the result is negative denoting the absence of Hemoglobin S.

Hemoglobin S is presently considered an inherited genetic trait which, when heterozygous gives little clinical evidence of its presence, but when homozygous results in profound anemia.

Sickle cell trait has been found predominantly in persons of the Negro race. Homozygous Hemoglobin S results in an anemia whose symptoms include leg ulcers and acute attacks of pain. The homozygous condition is normally distinguished by the presence of peculiar sickle-shaped and oat-shaped red corpuscles. Although it has been associated in the main with persons of the Negro race, there are indications that the sickle cell trait may have been introduced into Africa from the northeast via the former land bridge between Egypt and Africa. However, it appears that sickle-cell anemia presently is found more often in persons of the Negro race or persons having a mixture of Negro blood.

The doctor examining the patient who was afflicted with sickle-cell anemia and who did not keep that possibility in mind, without an appropriate blood examination, might make an error in diagnosis as the symptoms of sickle-cell anemia are also common to abdominal disease, rheumatic fever or a neurological disorder.

It is important that even sickle-cell trait be recognized by a treating physician, as a person having this trait should not be subjected to low oxygen conditions. Further, this is also important where one having sickle-cell trait gives blood for use by others. Certainly, it would not be desirable to give a transfusion of blood of one having Hemoglobin S to a patient in whom an oxygen deficiency might exist.

Various methods can be used to determine sickling. The most simple method is to place a drop of blood on a slide, apply a cover slip and seal the preparation. As the preparation stands, the oxygen is consumed. Sickling will be visible under a microscope in a few hours in the case of sickle-cell anemia, and more slowly with sickle cell trait and can be observed by a skilled technician. Sickling would be hastened by placing a rubber band tightly around the finger and leaving it in place about five minutes before making a wet preparation. Maximum sickling is produced by adding to the blood a reducing agent such as

2

sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ ) or sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ). The sodium metabisulfite is used for screening purposes in accordance with the following steps:

(1) Placing a small drop of capillary or oxalated blood on a clean slide and adding to the slide two drops of the same size of a 2 percent sodium metabisulfite solution.

(2) The blood and sodium metabisulfite solution is mixed and covered with a glass and allowed to stand for 30 minutes.

(3) By observing under a microscope (high dry lens), it is possible to recognize, with a skilled eye, the presence of sickle cells.

One qualitative test for distinguishing sickle-cell trait from sickle-cell anemia presently utilized is Sherman's method. This method utilizes a 10 percent Formalin solution. The steps comprise:

(1) Placing 2 ml. of the 10 percent Formalin saline solution in a small medicine glass covering with a layer of mineral oil.

(2) Fill the dead space of a 5 ml. syringe with mineral oil and expell the excess.

(3) Place a sterile needle on the syringe and withdraw 2 ml. of blood by venipuncture.

(4) Leave the needle on the syringe and immediately deliver one ml. of blood below the layer of oil in the medicine glass.

(5) Mix by stirring with a glass rod.

(6) After the mixture has been left standing for 10 minutes, a small amount of blood mixture may be removed with a capillary pipet.

(7) Then a drop of the mixture is placed on the slide and covered with a cover glass and examined for sickle-cells under the high power lens of a microscope.

The result is that one would observe that either there are no sickle cells or one would count erythrocytes and the percentage of sickled erythrocytes present. If there were 1 percent or less sickled erythrocytes, then sickle cell trait would be indicated. Sickle cell anemia invariably shows more than 1 percent, usually between 30 and 60 percent sickled erythrocytes.

Of course, hemoglobin electrophoresis is the most specific method for determining the presence of an abnormal hemoglobin, such as Hemoglobin S, and for distinguishing sickle cell anemia (homozygous) from sickle cell trait (heterozygous).

A third method of qualitatively analyzing the blood for percentage of sickle cells is described by C. A. J. Goldbreg in an article in Clinical Chemistry, vol. 3, No. 1, February 1957, pages 1-19 entitled "Identification of Hemoglobins" in which special blood preparations requiring the following are utilized:

Four or five milliliters of oxalated blood are placed into centrifuge tubes of 15 ml. capacity and centrifuged for ten minutes.

The supernatant is withdrawn and 10 ml. of saline is added to the packed cells.

This is stirred gently and again centrifuged for ten minutes. Again the supernatant is withdrawn and the cells are washed three more times with 10 ml. portions of saline.

This is again centrifuged for ten minutes.

The washed, packed cells are placed in graduated centrifuge tubes and two volumes of barbital buffer pH 8.6 are added with gentle stirring, and the suspensions are then transferred to freezer storage tubes. The samples frozen are maintained at least overnight and they may be kept in the frozen state until needed.

Hemolysis is effected by thawing. One tube is thawed in the refrigerator or at room temperature. The tube may be warmed at body temperature but should be cooled as

3

soon as all the ice has disappeared. The tube cannot be placed in warm or hot water to thaw.

The sample is centrifuged for ten minutes and the clear hemolysate is then usable for further testing.

A phosphate buffer is then prepared by dissolving 16.9 grams of monobasic potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) and 21.7 grams of dibasic potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) or 17.7 grams of dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) in carbon dioxide-free distilled water and the volume is adjusted to 100 ml.

1.8 ml. of the phosphate buffer, 20 mg. of sodium hydrosulphite and 2 ml. of the buffered hemoglobin solution are added to a small test tube.

The test tube is mixed and allowed to stand for fifteen minutes. A precipitate is formed which is then separated by filtering the solution through Whatman filter paper No. 5 or its equivalent.

3.8 ml. of the phosphate buffer and 20 mg. sodium hydrosulphite are measured into cuvettes. Then, .2 ml. of the hemoglobin filtrate is added and mixed by inverting the cuvettes twice. The absorbancy or optical density of the solution is then measured in a spectrophotometer at 415  $\mu$ .

Then, into a graduated cylinder of 25 ml. capacity, 20 ml. of distilled water are added, and .1 ml. of hemoglobin solution is placed in the cylinder. Then the pipet is rinsed and the cylinder is mixed by inversion. About 4 ml. of the solution is transferred to a cuvette.

When the absorbancy of the control solution is measured in accordance with the following formula:

$$\text{Solubility percent} = \frac{A_{\text{unknown}}}{A_{\text{control}}} \times 100$$

The solubility of Hemoglobins A and F has been found to be 90 percent or higher by this method. The solubility of Hemoglobin S is very low.

All three of the qualitative methods set forth above, namely Sherman's method, electrophoresis and the Goldberg method have essential drawbacks. They take a very long time to complete (Goldberg's method would take at least 24 hours) and require skilled technicians to both perform the method and to recognize the results. Thus, none of these three methods would be suitable for screening large numbers of blood samples in a short time by unskilled personnel. Further all of the above procedures require expensive equipment.

The only method suggested in the past for use as a screening test and in fact, utilized as a screening test, is the metabisulfite test discussed above. However, this test, too, requires at least 30 to 35 minutes to complete and additionally requires a trained person to observe the results. That is, the person must be able to use a microscope to recognize sickle cells as opposed to other normal cells and other abnormal blood cells which may resemble sickle cells. In the case of sickle cell trait, this sometimes can be a problem as the number of sickle cells present on the slide may be few and the viewer can miss the telltale shape of the cells, thus giving a negative result. It will easily be understood that to miss a positive result, i.e. the presence of sickle cells, can be extremely dangerous. Sometimes the sickle cell trait is not observable within 30 minutes and the blood must be reexamined after a substantially longer period of time.

Another important problem with certain of the tests is that they require either large amounts of blood or they cannot use whole blood. In those places where there are no elaborate facilities for processing blood, it may not be possible to utilize certain of the above tests and it may be necessary to send the blood, obviously in a large quantity, to a central testing laboratory where the test will be performed.

#### Summary of the invention

The present invention is intended to be a two-minute test for detecting the presence of Hemoglobin S. The test

4

requires only 0.02 milliliter of whole blood and therefore, can be performed with a drop of blood. Further, it can be performed and evaluated by unskilled personnel.

Venous blood is not required and the test can utilize blood obtained from a pin prick. This test utilizes the same phenomena noted in Goldberg's test, namely, that Hemoglobin S has a lower ferrohemoglobin solubility as distinguished from all other hemoglobins except perhaps Hemoglobin H. However Hemoglobin H is so rare a condition as to be of negligible importance. Further, since the test is only used as a screening test, while this test shows Hemoglobin S to be present, it is normally followed by one of the quantitative tests discussed above and most probably electrophoresis, to determine the exact composition of the blood. Thus, in discussing ferrohemoglobin solubility, it can be said that Hemoglobin S, in its reduced form is insoluble in a phosphate buffer in the presence of sodium hydrosulfite. A phosphate buffer is prepared, which in the preferred embodiment was formed from 16.9 grams of monobasic potassium phosphate, and 21.7 grams of dibasic potassium phosphate which are diluted with distilled water to a volume of 100 ml. The phosphate buffer has a high hydrogen ion concentration and the pH of the solution is between 6.5 and 6.8. To the phosphate buffer solution is added 6 grams of sodium hydrosulfite. The sodium hydrosulfite is dissolved in the phosphate buffer solution by swirling or by a vortex method of mixing.

A rapid erythrocytic hemolyzing agent that is capable of inducing multiple lesions of the erythrocyte membrane is then added to the resultant solution. In the preferred embodiment 10 ml. of a 2 percent saponin solution in isotonic sodium chloride ( $\text{NaCl}$ ) was added to the previously formed solution. The resultant solution was then mixed and dispensed into 10 x 75 mm. tubes with 2 ml. of the solution being placed in each tube.

.02 ml. of whole blood is then placed in one of the 10 x 75 mm. tubes and mixed by vortex or lateral swirling or by multiple inversions. Then, one merely waits for a maximum of 2 minutes. If the solution in the 10 x 75 mm. tube has any cloudy or turbid look caused by a precipitate, this is a positive indication of the probable presence of Hemoglobin S. If the solution in the 10 x 75 mm. tube is opalescent, reddish and translucent, the result is negative and there is no Hemoglobin S in the blood being tested. As an aid in detecting the results, one could place a white card with a black line behind the test tube. If, after the two minutes had elapsed, one could see the black line, the result of the test would be negative. If one could not see the black line, the result is positive meaning that there is a high probability of the presence of Hemoglobin S. The result is dramatic and the white card is not ordinarily necessary for detecting the results of the test. Obviously, it will be understood that the solution prepared in accordance with the above method would fill over 50 test tubes and thus 50 tests can be performed. It has been found that the solution with the erythrocytic hemolyzing agent can be kept at least six weeks under refrigeration and that the solution can be kept for even longer periods of time when the erythrocytic hemolyzing agent and reducing agent are kept separate.

The tests can be further modified by merely placing a drop of the buffer, reducing agent, and hemolyzing agent solution on a glass plate and, then placing less than a drop of blood from an applicator stick in the solution. When the glass plate is held over a background having a line passing through positions underneath the place where the test is performed, the line, in two minutes, will either be visible if the result is negative or will be blocked by the turbidity of the solution and blood mixture when Hemoglobin S is present. This would allow for mass screening of blood in the most simple and elemental form. It will further be understood that both of the tests outlined above can be performed by persons totally unskilled in the art of blood testing as they need merely drop .02 ml. of whole blood into a pre-prepared solution and visually determine

whether the resultant solution is translucent or turbid thus determining the absence or probable presence of Hemoglobin S.

For the purpose of illustrating the invention, there are shown in the drawings forms which are presently preferred; it being understood, however, that this invention is not limited to the precise arrangements and instrumentalities shown.

FIGURE 1 is a front view of testing apparatus utilizing the principles of the present invention prior to the addition of whole blood.

FIGURE 2 shows the apparatus of FIGURE 1 after whole blood having Hemoglobin S therein has been added to the testing solution giving a positive result.

FIGURE 3 is a top plan view of a glass plate utilized for mass screening of bloods to determine the presence or absence of Hemoglobin S.

As was discussed previously, the basic invention is practiced by preparing a phosphate buffer solution discussed previously, that is, mixing 21.7 grams of dibasic potassium phosphate ( $K_2HPO_4$ ) with 16.9 grams of monobasic potassium phosphate ( $KH_2PO_4$ ) and diluting with distilled water to 100 ml.

A reducing agent in the form of 6 grams of sodium hydrosulfite is then mixed with the potassium phosphate buffer and dissolved in the buffer by swirling or other suitable means.

The hemolytic agent is then added to the phosphate buffer and reductant solution. Many types of hemolytic agents are available. However, a preferred hemolytic agent is a 2 percent saponin solution in isotonic sodium chloride. This has been found to be especially effective for rapid hemolyzing. Saponin ( $C_{32}H_{52}O_{17}$ ) is a term applied to two groups of plant glucosides that have the ability to hemolyze red corpuscles at very great dilutions. The rapidity of hemolysis utilizing these saponins depends to a large extent on the plants from which a particular saponin is produced, on the purity of the saponin and possibly even on the place where the particular plant was grown. It has been found that the saponins manufactured by Glenwood Chemical Company, 83 Summit St., Tenafly, N.J., under the trademark Sapolysin and by Coulter Electronics Company of Hialeah, Fla. are effective in the process of the present invention. However, other saponins will be effective in accordance with the teachings of the present invention except that those with lesser hemolytic effects will, of course, take longer to dissolve the red cell membrane and release the hemoglobin. Then the waiting time will be extended beyond the two minutes set forth below.

The 10 ml. of the 2 percent saponin solution in isotonic sodium chloride is mixed with the phosphate buffer and reducing agent solution. Then, the resultant solution is dispensed into 10 x 75 mm. test tubes in quantities of 2 ml. As an aid in detecting the end point of the test, the test tubes 10 with the test solution 12 as shown in FIGURE 1 are placed in front of a test card 14. The card 14 has a black line 16 running horizontally across the center thereof. As can be seen, the test line 16 will be visible through the solution 12 and the test tube 10. A Sahli pipet (.02 ml.) 18 is utilized to supply .02 ml. of whole blood into the test tube 10 to mix with the solution 12. The blood from pipet 18 and the solution 12 are then mixed by vortex, lateral swirling, or by multiple inversions. Then the test tube is allowed to stand for approximately 2 minutes.

At the end of 2 minutes, if, as shown in FIGURE 2, the solution 12' including the blood and the original test solution 12 is now cloudy, or turbid resulting from the precipitate caused by reason of the insolubility of Hemoglobin S in a phosphate buffer in the presence of sodium hydrosulfite, then the result is positive indicating the probable presence of Hemoglobin S. The turbidity and cloudiness of the solution is easily seen by the fact that the line 16 is no longer visible through the new solution 12' and test tube 10. If the line 16 is visible,

this means that the result is negative and there is no Hemoglobin S present. The negative result can also be seen by observing that the resultant solution 12' is opalescent, reddish, and translucent.

A method of mass screening of blood samples for Hemoglobin S is shown in FIGURE 3. A drop of the solution 12 of FIGURE 1 comprising the mixture of phosphate buffer, reducing agent, and hemolytic agent is placed in various spaces 20 on a glass or transparent plate 22. Then the glass plate 22 is placed on a background 24 having lines 26, 28 and 30 running horizontally along the width thereof. Samples of blood are then added by an application stick which has been dipped in blood and stirred in the solution on the positions 20. After 2 minutes, each of the positions 20 is observed. If the lines 26, 28 or 30 associated with a particular position 20 can be seen through the test solution blood mixture, then the test result is negative. If the particular line 26, 28 or 30 associated with a position 20 is not visible through the blood-test solution mixture, then the result is positive.

It will be understood that the buffer solution set forth above can be formed by dissolving 17.7 grams of dibasic sodium phosphate in carbon dioxide-free distilled water and the remainder of the process would be the same to produce similar results.

I claim as my invention:

1. A method of testing blood for the presence of Hemoglobin S comprising the steps of providing a high ionic concentration buffer solution, adding a reducing agent to the buffer, adding an erythrocytic hemolyzing agent to the buffer and reducing agent solution, and then adding a measured amount of blood to the buffer, reducing agent and hemolyzing agent solution after mixing the same, and, after a predetermined amount of time, observing the resultant solution to determine, by the turbidity of the resultant solution, either the absence or probable presence of Hemoglobin S in the blood.

2. The method of testing blood for the presence of Hemoglobin S of claim 1 wherein said erythrocytic hemolyzing agent is a saponin solution.

3. The method of testing blood for the presence of Hemoglobin S of claim 1 wherein said reducing agent is sodium hydrosulfite.

4. The method of testing blood for the presence of Hemoglobin S of claim 1 wherein said test solution is formed in the same proportions as is achieved by providing 100 ml. of a mixture of the phosphate buffer and reducing agent and 10 ml. of a saponin solution the concentration of which depends on its erythrocytic hemolyzing ability.

5. The method of testing blood for the presence of Hemoglobin S of claim 1 wherein said erythrocytic hemolyzing agent is a saponin solution of a low concentration.

6. The method of testing blood for the presence of Hemoglobin S of claim 1 wherein the buffer agent, reducing agent and erythrocytic hemolyzing agent solution is placed in a transparent container and .02 ml. of whole blood is added thereto.

7. The method of testing blood for the presence of Hemoglobin S of claim 1 wherein said high ionic concentration buffer is a phosphate buffer.

8. The method of testing blood for the presence of Hemoglobin S of claim 7 wherein said buffer solution includes dibasic sodium phosphate.

9. The method of testing blood for the presence of Hemoglobin S of claim 7 wherein said buffer solution includes a mixture of dibasic potassium phosphate and monobasic potassium phosphate at a high hydrogen ion concentration.

10. The method of testing blood for the presence of Hemoglobin S of claim 9 wherein the step of providing a buffer and adding a reducing agent thereto is achieved in the proportion as would be achieved by mixing 21.7

grams of dibasic potassium phosphate and 16.9 grams of monobasic potassium phosphate to distilled water to a total volume of 100 ml. and adding the reducing agent in the form of 6 grams of sodium hydrosulfite to the phosphate buffer solution.

11. A product for testing of whole blood for Hemoglobin S comprising a high ionic concentration buffer solution mixed with a reducing agent and an erythrocytic hemolyzing agent.

12. The product for testing of whole blood for Hemoglobin S of claim 11 wherein said high ionic concentration buffer solution is a phosphate buffer solution.

13. The product for testing whole blood for Hemoglobin S of claim 11 wherein said buffer is a dibasic sodium phosphate solution.

14. The product for testing whole blood for Hemoglobin S of claim 11 wherein the erythrocytic hemolyzing agent is an aqueous saponin solution.

15. The product for testing of whole blood for Hemoglobin S of claim 14 wherein said saponin solution is a 2 percent saponin solution.

16. The product for testing whole blood for Hemoglobin S of claim 15 wherein the erythrocytic hemolyzing agent solution comprises 10 percent of the volume of the high ionic concentration buffer and reducing agent solution.

17. The product for testing whole blood for Hemoglobin S of claim 11 wherein said reducing agent is sodium hydrosulfite.

18. The product for testing whole blood for Hemoglobin S of claim 17 wherein said buffer is a mixture of monobasic potassium phosphate and dibasic potassium phosphate in solution.

19. The product for testing whole blood for Hemoglobin S of claim 18 wherein said monobasic potassium phosphate is by weight to said dibasic potassium phosphate as 16.9 is to 21.7.

#### References Cited

##### UNITED STATES PATENTS

2,519,997	8/1950	Brown	23—230	XR
3,000,836	9/1961	Ginsburg	252—408	
3,374,063	3/1968	Noller	23—230	
3,446,751	5/1969	Weichselbaum	23—230	XR

##### OTHER REFERENCES

Miller, Seward: A Textbook of Clinical Pathology, Sixth edition, The Williams & Wilkins Co., Baltimore, 1960; pages 115—120 relied on.

MORRIS O. WOLK, Primary Examiner

B. S. RICHMAN, Assistant Examiner

U.S. Cl. X.R.

252—408; 424—101