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[54] **DIAGNOSTIC TEST FOR THE DETERMINATION OF SICKLING HEMOGLOBINOPATHIES**

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[58] **Field of Search** .... 23/230 B; 252/408; 424/101

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[57] **ABSTRACT**

A novel composition comprising an aqueous and an organic phase, the aqueous phase comprising essentially a phosphate buffering system containing a stable reducing agent and a lysing agent, the composition providing for a test for the determination of sickling hemoglobin that is simple to perform, is stable, convenient, reliable, fast and one that differentiates between heterozygous Hemoglobin S and homozygous Hemoglobin S without false positives or false negatives.

9 Claims, No Drawings

DIAGNOSTIC TEST FOR THE DETERMINATION OF SICKLING HEMOGLOBINOPATHIES

This invention relates to a novel screening composition suitable for accurately and reliably determining the presence of sickling hemoglobin and for accurately and easily determining whether the patient having sickling hemoglobin is heterozygous or homozygous.

As is well known, it is essential in clinical diagnosis to determine the presence of Hemoglobin S and, once so-determined, to detect whether the patient has sickle-cell anemia (i.e., SS or homozygous) or sickle-cell trait (i.e., AS or heterozygous). As is also well known, several systems for this determination have been developed but such prior art systems have, in one aspect or another, failed to meet the desired criteria for the efficient applicability for both field and laboratory use. It is believed that the composition of the instant invention does meet the criteria for such uses and offers such advantages that neither the laboratory nor the field technicians need prepare his own reagent. In essence, the procedure facilitates unit packaging at a manufacturer's level, is simple and rapid, provides for reagent stability of up to 2 months, has an endpoint stability which provides for reading the results even after periods of up to 6 weeks, does not require the use of any viewing devices and affords the presumptive identification of normal (AA), trait (AS), and disease (SS) hemoglobin genotypes with an increase in reliability. Additionally, the instant invention has possible application for the determination of such blood characteristics in newbrons. Thus, the composition has value for rapidly screening samples newborns. routine practice, for medical emergencies and in the practice of pediatric medicine.

In general, the composition is comprised of an aqueous phase containing a phosphate buffer system, a stable reducing agent system and a lysing agent, said aqueous phase being in combination with an organic phase which is immiscible with the aqueous phase.

More specifically, this invention relates to a two-phase composition comprised of an aqueous phase and an organic phase wherein the aqueous phase is comprised of about 38–45 percent solids (on a weight-/volume basis) having a hydrogen ion content sufficient to provide a pH value of about 6.3 (±0.15), said solids being comprised of (a) a lysing agent providing for up to about 2 percent of the total solids content (b) a mixture of chemical reducing agents providing for up to about 22.4 percent of the total solids content and (c) a phosphate ion buffer system providing the remaining total solids (75.6 percent) present in the aqueous phase. The phosphate ion is provided by dibasic alkali metal phosphate (preferably dibasic potassium phosphate) and monobasic alkali metal phosphate (preferably monobasic potassium phosphate) in the weight/-weight ration of 1.75 of the dibasic phosphate to 1.0 of the monobasic phosphate. The organic phase, added after the aqueous phase has been aliquoted, is preferably in the volume/volume ration of 1 part organic phase to 4 parts aqueous phase. Although minor variations are possible, it is preferred to employ a total solids content in the aqueous phase within the range of 38–45percent solids, with 41 percent being most preferred. Upwards variations in the total solids of about 10 percent over the foregoing range gives false positives, while downward variations of about 10 percent of the foregoing range will give false negatives. The most preferred

stable chemical reducing system is provided for by combining sodium hydrosulfite and sodium bisulfite in the sodium hydrosulfite/sodium bisulfite ratio of 1/10 (wt./wt.).

The preparation of the most preferred formulation is effected by adding (a) 237 gms. of dibasic potassium phosphate, (b) 135 gm. of monobasic potassium phosphate, (c) 10 gm. of sodium hydrosulfite and (d) 100 gm. of sodium bisulfite to 1000 ml. of distilled water (maintained at room temperature), stirring the resulting mixture until all ingredients are dissolved, filtering through a Whatman filter paper (No. 1), and then adding 10 gms. of saponin to the filtered solution and stirring that mixture until dissolution is effected. The resultant solution is then dispensed in 2 ml. aliquot portions into 10 × 75 mm. screw cap clear test tubes to which is then added 0.5 ml. of the organic phase, preferably toluene.

As noted, the foregoing formulation is the most preferred. Other organic solvents capable of forming an immiscible organic phase on top of the liquid phase may also be used, among which solvents are n-pentane, hexane, heptane, octane, nonane, decane, undecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, cyclopentane, cyclohexane, cycloheptane, cyclooctane, allyl chloride, toluene, ethylbenzene, n-propylbenzene, cumene, (isopropyl benzene), tert. butyl benzene, o-xylene, m-xylene, and p-xylene, (It should be noted that such organic solvents (especially toluene) may also be used as lysing agents, although saponin is still most preferred.)

Other reducing agents such as sodium metalbisulfite, cystine hydrochloride, ascorbic acid, hydrogen sulfide, 2,3-dimercapto propanol, glutathione, methylene blue, potassium ferrocyanide, thioglycolate, lactic acid and succinic acid may also be used. With the exception of the first three, most others are substantially less effective. The literature also suggests other lysing agents (e.g. digitonin, sodium lauryl sulfate, Tween 20, ethylhexadecyl methyl-ammonium bromide, palmitoyl-D, L-carnitine, palmitoyl choline and (1-palmitoyl), lysolecithin and many others, but saponin is most effective.

Once prepared, the composition is very easily used. In practice, each tube contains sufficient reactants for a single test and is stable for at least six months, both at room temperature and at 4°C. This combination of features, of course, allows for both convenience and lack of waste, thereby reducing the overall cost of testing.

To perform the test, a 100 microliter sample of blood (2–3 drops) is added to the reagent and the mixture shaken vigorously for a few seconds. After allowing for the chemical reduction of the hemoglobin (i.e. the hemoglobin turns reddishpurple after about 3 minutes) the tube is centrifuged. Preferably, centrifugation is at 3500–5000 r.p.m. for at least 2 minutes. Slower speeds for longer periods (i.e. 2500 r.p.m. for 5 minutes) may also be employed.

Of course, the centrifugation is nothing more than a method of phase separation. In view of the stability afforded by this test procedure, this phase separation need not be accelerated by centrifugation. In such instances the shaken mixture is allowed to stand until the phase separates, which generally takes about within 1–2 hours. The foregoing phase separation results in a two-layer system separated by a band in the middle; the aqueous phase is the lower phase and the organic layer is the upper phase. The upper organic phase is immiscible with the aqueous phase and serves to bind sickling

hemoglobin to the liquid/liquid interface. This physical affinity is so strong that tipping over the tube (or inversion of the tube) often fails to disrupt the final pattern formed after phase separation. On those random occasions wherein a pattern is accidentally disrupted, it can be re-separated. Of course, in each instance, it is most preferred to accelerate the phase separation by the above-described centrifugation technique, non-use being preferably limited to those situations wherein a centrifuge is unavailable. Experience has shown that those samples which are centrifuged produce a very tight, more easily recognized, band at the interface as well as producing a more stable two-phase system.

The blood sample used can be fresh whole blood, whole blood with added anticoagulant, or hemoglobin preparations, either fresh or stored for several months. Preferably, the blood is obtained from a pricked-and-bled finger.

The results are read as follows:

Hemoglobin Genotype	Middle Band	Lower Layer
AA	Grey	Red
AS	Red	Pink
SS	Red	Straw colored or pale yellow

With normal blood the middle band is grey and the lower phase is red. When the hemoglobin genotype is AS the middle band is dark red and the lower phase is pink. When the hemoglobin genotype is SS the middle band is dark red and the lower phase is straw-colored or pale yellow.

We claim:

1. A method for testing blood for the presence of Hemoglobin S which comprises vigorously admixing a blood sample with a two-phase test system and determining the presence or absence of Hemoglobin S after separation of the phases, said two-phase test system comprising (a) an aqueous phase containing on a weight/volume basis of about 38–45 percent total solids, said total solids consisting of a lysing agent in amounts up to about **2** percent of the total solids, a chemical reducing system in amounts up to about 22.4 percent of the total solids and a phosphate ion buffer system in amounts up to about 75.6 percent of the total solids, and (b) an organic phase immiscible with and lighter than said aqueous phase; the Hemoglobin S characteristic being determinable by the color of a band formed at the interface of the organic and aqueous phases and the hemoglobin genotype AS being determinable from the hemoglobin genotype SS by the color of the aqueous phase.

2. A process according to claim **1** wherein the test procedure forms a distinguishing band at the interface of the two phases after a blood sample has been vigorously admixed with the test system.

3. A method according to claim **1** wherein the phosphate buffer system is present in the aqueous phase in the amount of about 31 percent (weight/volume).

4. A process according to claim **1** wherein the phosphate ion buffer system consists of dibasic potassium phosphate and monobasic potassium phosphate in weight/weight proportions of 1.75/1.0, respectively, said aqueous phase having a hydrogen ion concentration capable of providing a pH of about 6.3 (**±0.15**).

5. A composition suitable for testing whole blood for Hemoglobin S comprising (a) an aqueous phase having a total solids content (weight/volume) of about 38–45 percent and (b) an organic phase immiscible with and lighter than said aqueous phase, said aqueous phase capable of producing a hydrogen ion content sufficient to effect a pH of 6.3 (±0.15), said aqueous phase containing a phosphate ion buffer system in amounts up to about 75.6 percent of the total solids content, a lysing agent in amounts up to about 2 percent of the total solids content and a chemical reducing system in amounts up to about 22.4 % of the total solids content.

6. A composition of claim **5** wherein said lysing agent is saponin.

7. A composition of claim **5** wherein said organic phase is toluene.

8. A composition of claim **5** wherein the chemical reducing system is composed of sodium bisulfite and sodium hydrosulfite, said sodium bisulfite being present in amounts up to about ten times as much as the amount present of sodium hydrosulfite.

9. A composition of claim **5** wherein the dibasic potassium phosphate is present in amounts up to 1.75 times the amount of monobasic potassium phosphate.

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