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LYOPHILIZED DIAGNOSTIC REAGENT FOR THE DETERMINATION OF BLOOD COAGULATION FACTORS

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ABSTRACT OF THE DISCLOSURE

This invention discloses a lyophilized diagnostic reagent for the determination of blood coagulation factors. Broadly, this composition comprises a blood platelet factor substitute lyophilized together with finely-divided, discrete, inert particles.

This invention relates to novel compositions of matter. More particularly, this invention relates to compositions comprising a blood platelet factor reagent which contains a Hageman Factor activator and which is valuable for use as a diagnostic aid for the determination of blood coagulation factors. This invention also includes within its scope a novel process for the production of these compositions as well as methods for their use.

The mechanism of blood coagulation is complex but according to Miale, Laboratory Medicine & Hematology (1962) published by the C. V. Mosby Company, this mechanism may be considered as phenomenon resulting from the interaction of four plasma components:

- (1) prothrombin
- (2) thromboplastin
- (3) thrombin, and
- (4) fibrinogen

in the presence of calcium ion to form a clot or fibrin. If there are any deficiencies in these components or any defect in their interaction, such abnormalities will be reflected in an altered blood coagulation picture which may be manifested by a failure to clot, under prolongation of clotting time, poor clot retraction and so on. Clinically, these abnormalities are manifested by syndromes such as hemophilia, purpura, afibrinogenemia and the like. In order to facilitate the diagnosis of these diseases, many laboratory tests have been devised to detect and diagnose the defective factor or factors so that proper preventive measures may be initiated or prior warning obtained when surgery is contemplated. These tests include, for example, the Duke method to determine bleeding time, the Lee-White method for determining clotting or coagulation time, and the Quick method to determine prothrombin. Recently, the partial thromboplastin time test, described by Langdell et al., J. Lab. & Clin. Med. 41:637, 1953, has been widely recognized as a test procedure for testing the coagulation mechanism as a whole. This test determines the defects which may exist in all plasma clotting factors except platelet factor and Factor VII. This test is based on the theory that plasma thromboplastin is formed in the presence of platelet factor and in the presence of the following plasma factors viz: antihemophilic factor (AHF), Plasma Thromboplastin Component (PTC), Factor X, Factor XI or Plasma Thromboplastin Antecedent (PTA), Hageman Factor or Factor XII, calcium ion and Factor V.

Deficiencies or abnormalities in any one of the above factors leads to abnormal thromboplastin formation and this is reflected in the overall clinical coagulation picture.

In carrying out the test of partial thromboplastin time, the patient's plasma is mixed with a standardized reagent

containing a component which functions in the presence of optimum calcium ion concentration as platelet factor and thereby acts as a substitute, and the clotting time observed is noted. The platelet factor substitute is generally obtained as described below from warm-blooded animal brain tissue and is essentially cephalin having platelet factor-like activity. While this test is a valuable tool for the study of the generation of plasma thromboplastin and the diagnosis of various hemorrhagic disorders due to defects at this level, it has been observed that this test is very sensitive to certain environmental conditions. Thus, for example, if one carries out the test in glassware which has uneven surfaces this tends to accelerate the clotting time whereas the use of glassware having smooth surfaces, on the other hand, tends to give a rather extended end point. Obviously, for the test to be meaningful, the end-point should be reproducible, sharp and not so readily influenced by such environmental factors.

Investigations into these phenomena and experiments with normal plasma having pre-determined clotting time have ascertained that the presence of dust particles or of scratched glassware surface is responsible for the activation of the Hageman factor in the plasma whereas in the absence of such foci the action of the Hageman factor is slowed and the clotting mechanism therefore takes longer to act. Thus, even though none of the plasma factors are deficient, the results observed are variable and it appears that Hageman factor still needs to be activated to insure proper clotting activity. Clearly, then, while a platelet factor reagent is required in the clotting mechanism, the composition should also contain an activator which while insensitive to environmental conditions will activate the Hageman factor present and thereby insure uniform clotting action.

Accordingly, a primary object of this invention is to provide a modified platelet factor reagent containing an added Hageman factor activator of predictable action.

A further object of this invention is to provide a platelet factor reagent containing an activator and which will be useful as a reagent for the accurate determination of all plasma coagulation factors except Factor VII.

Another object of this invention is to provide a stable platelet factor reagent system containing an activator which will activate Hageman factor and which when used in coagulation assays gives a high order of reproducibility.

Other objects and advantages of this invention will become more apparent from the following detailed description.

Broadly speaking, the novel composition of this invention is a lyophilized composition which contains as the active ingredients a platelet factor reagent in combination with a Hageman factor activator of precise and controlled activity.

The platelet factor reagent may be obtained, for example, by extracting warm-blooded mammal brain tissue such as human or rabbit brain in accordance with the procedure described by Bell and Alton in Nature 174:880-881 (1954). To the brain extract thus obtained is then added an activator in particulate form suspended in a buffer system having a pH of about 7.5 and the resulting mixture is then lyophilized. The particulate substance employed as the activator should be inert, resuspend very rapidly and have a uniform particle size and should not have too alkaline or too acid a reaction to avoid any interference with the assay.

As examples of suitable activators which may be employed, there may be mentioned, for example, finely-divided diatomaceous earth, bentonite, kaolin, sand and powdered glass, and having a particle size of about from 2 to 20 microns.

In particular, an analytical grade of diatomaceous earth having a particle size of from 2 to 10 microns and marketed commercially by Johns-Manville Co. as "Celite 505" is preferred for use as the activator because it is inert, uniform in size, resuspends readily and is essentially neutral in reaction.

For optimum results, we mix about 0.5 to 3% by weight of activator with each 100 ml. of the platelet factor extract. In order to provide a uniform suspension when the lyophilized material is reconstituted in an aqueous solvent, it has been found to be advantageous to include about 0.5 to 2% by weight of a suspending agent in the mixture undergoing lyophilization. Suspending agents such as calcium-free acacia, calcium-free tragacanth, calcium-free guar gum and the like are particularly advantageous.

In order to further illustrate this invention, the following examples are given:

Example 1

About 5 fresh rabbit brains are processed in accordance with the Bell et al. procedure, supra. Briefly, the rabbit brains are ground up in a mortar with about 50 ml. of dry acetone until they are finely broken up. The acetone solution is decanted and the same process of grinding and washing with dry acetone is repeated until the acetone washings are free of cholesterol, as indicated by a negative Liebermann-Burchardt reaction. The acetone dried tissues are then extracted with approximately 50 to 100 cc. of chloroform. The chloroform extracts are carefully evaporated to give a residue of from about 300 to 400 mg. This residue when homogenized in about 125 ml. of aqueous isotonic sodium chloride solution forms the desired extract.

The aqueous rabbit brain extract thus formed is blended with 32.5 grams of Celite 505 in about 3.1 liters of an aqueous solvent medium comprising 30 grams of calcium-free acacia gum, 21 grams sodium barbital, and 15 grams of sodium chloride to obtain a uniform suspension. The pH of the suspension is then adjusted to 7.5 with 10% aqueous hydrochloric acid. About 2.5 ml. of the resulting mixture is filled into individual vials or ampules and the material lyophilized, the lyophilized product constituting the desired blood platelet factor reagent.

Example 2

To employ the lyophilized blood platelet factor reagent composition obtained in accordance with Example 1, the lyophilized unit in an individual ampule is first reconstituted in about 2.5 ml. of deionized water. An aliquot portion of the aqueous composition is mixed with an equal volume of 0.025 molar calcium chloride solution. A standard human plasma, such as "Diagnostic Plasma" marketed by Warner-Chilcott which is essentially a pooled lyophilized normal human plasma standardized to contain optimal concentrations of all clotting factors is reconstituted in deionized water. 0.1 ml. of this reconstituted aqueous plasma is blown into a test tube containing 0.2 ml. of the reconstituted platelet factor reagent containing the activator and calcium chloride mixture and the latter maintained at 37° C. This mixture is prepared in triplicate. As the plasma is added, the action of an electric timer is started. The elapsed time until the first formation of fibrin strands is observed is considered to be the end point. The elapsed time for the respective tests until fibrin strand formation is observed is the following:

- (1) 36.5 seconds
- (2) 36.0 seconds
- (3) 36.7 seconds

When the above test is repeated employing the same platelet factor reagent but without the activator, elapsed time for the first fibrin strand formation in the respective tubes is:

- (1) 83.5 seconds
- (2) 76.8 seconds
- (3) 72.1 seconds

A human plasma which is known to be deficient in Factor VIII is subjected to the same partial thromboplastin test described above. The elapsed time until fibrin strand formation is observed, employing the novel composition of this invention, is as follows:

- (1) 71.6 seconds
- (2) 69.9 seconds
- (3) 73.1 seconds

The same test is repeated employing the same platelet factor reagent but without the activator. The elapsed time for the first fibrin strand formation in the respective tubes is:

- (1) 180.2 seconds
- (2) 200.8 seconds
- (3) 168.5 seconds

The above results overwhelmingly indicate the reproducibility, sharpness and accuracy of the test as well as the highly advantageous rapidity of the test when employing the novel compositions of this invention in the partial thromboplastin time test.

It is to be understood that the foregoing detailed description is given merely by way of illustration and that many variations may be made therein without departing from the spirit of our invention.

Having described our invention, what we desire to secure by Letters Patent is:

1. A lyophilized composition of matter suitable for reconstitution by addition of water comprising blood platelet substitute selected from the group consisting of human and rabbit brain chloroform extracts in combination with 0.5 to 3 parts by weight of finely-divided discrete inert particles selected from the group consisting of kaolin, powdered glass, bentonite, and diatomaceous earth and a suspending agent selected from the group consisting of calcium-free acacia, tragacanth, and guar gum, said composition being buffered to a pH of about 7.5.

2. A composition as defined in claim 1, wherein said particles are from about 2 to 20 microns in size.

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