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3,228,841

DIAGNOSTIC REAGENT COMPOSITION FOR DETERMINING BLOOD COAGULATION FACTORS AND METHOD OF USE**Raphael Cohen, Morristown, and Jane G. Lenahan, Florham Park, N.J., assignors to Warner-Lambert Pharmaceutical Company, Morris Plains, N.J., a corporation of Delaware**

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This is a continuation-in-part application of copending application Serial No. 136,749 filed September 8, 1961, now abandoned.

The present invention relates to a novel diagnostic composition and more particularly to a novel lyophilized diagnostic composition useful for the determination of blood clotting factors.

Anticoagulant therapy is primarily designed to prevent intravascular clotting. The indications for the prophylactic and therapeutic uses of anticoagulants such as heparin or bishydroxycoumarin include many medical and surgical conditions, for example, in pulmonary embolism to prevent further embolism which may prove fatal, in thrombophlebitis to forestall further venous thrombosis, and in various cardiovascular diseases such as congestive heart failure, coronary arterial occlusion, arterio-sclerosis and the like to lessen the probability of further attacks. In order to monitor anticoagulant activity the patient's plasma is constantly assayed to determine the clotting time so that dosages of anticoagulant drugs administered can be adjusted accordingly. The methods and their modifications that are employed in clinical laboratories for determining clotting time are, for example, Quick's one-stage method (Quick, A J., Am. J. Med. Sciences 190:501-11, 1935) and its modifications thereof and prothrombin-proconvertin test (P—P method) (Owren et al., Scand. J. Clin. and Lab. Invest. 3:201, 1951) and its modifications thereof. The basic procedure in all these methods or their modifications are the same—patient's plasma is added to a thromboplastin-calcium mixture and the time elapsed for the formation of fibrin or a clot is measured.

The thromboplastin is generally obtained from various mammalian tissue. It is essential in these tests that the plasma tested be fresh and all the glassware must be scrupulously clean and free from scratches. If the plasma is not freshly collected or the glassware is scratched the end point is not well defined. Generally speaking, the clotting time obtained with samples which are 24 hours old is longer than the clotting time observed with freshly obtained samples using these known methods. Since the dosages of anticoagulant administered depends very much on the determination of an accurate clotting time, the requirement that fresh blood samples be used and that the glassware be scratch-free poses many serious problems. Thus, for example, patients who live a long distance away from a clinical laboratory are unable to mail in their blood samples. This is clearly inconvenient for patients on long term anticoagulant therapy. On the other hand, the laboratory must perform the tests immediately after collection. Of course, the requirement of scratch-free glassware adds another factor to the proper performance of this important diagnostic test. Furthermore, it has been found that commercially available thromboplastin-

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calcium mixture is generally not stable and this also contributes to the difficulty in performing these unusually sensitive tests.

Accordingly, a primary object of this invention is to provide a stable diagnostic reagent which is not effected by environmental changes for determining blood clotting factors.

Another object of this invention is to provide a novel thromboplastin-calcium reagent which can be used in modifications of all known methods for determining clotting time.

A further object of this invention is to provide a lyophilized reagent containing all the factors not being assayed for in the patient's plasma for determining clotting time.

Yet another object of this invention is to provide a diagnostic reagent for determining clotting time which is not effected by the age of the patient's plasma.

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

According to this invention this novel diagnostic composition is obtained by combining tissue thromboplastin having strong activity toward human plasma, a source of calcium ion and plasma containing a high concentration of Factor V and fibrinogen and free of Factors II, VII, IX and X.

The tissue thromboplastin having strong activity toward human plasma is preferably obtained by combining equal parts by weight of rabbit lung tissue and rabbit brain tissue thromboplastin. These tissue thromboplastin fractions are prepared in the following manner. Brain tissue from freshly killed rabbits is dried and then pulverized in the presence of an excess of acetone. The brain tissue is combined with an equal weight of ground rabbit lung tissue which has been dried by a freeze-drying technique as described in "Biological Applications of Freezing and Drying" by R. J. Harris, Academic Press, New York. The combined rabbit tissues are extracted with an aqueous solution at 45°–50° C.

The plasma fraction containing a high concentration of Factor V and fibrinogen is generally obtained from bovine sources. Broadly speaking, bovine plasma is treated with a 10% by weight solution of barium sulfate by gentle agitation of the mixture followed by removal or barium sulfate by centrifugation. The treatment of plasma with barium sulfate results in the adsorption of Factors VII, IX, X and prothrombin by barium sulfate thereby giving optimum concentrations of Factor V and fibrinogen. The adsorbed bovine plasma and the aqueous extract of rabbit brain and lung thromboplastin fractions are then added to an aqueous solution containing 0.0125 M calcium chloride and 0.075 M sodium chloride. The mixture obtained is then buffered with a buffer such as a veronal buffer to give a final pH of 7.2 to 7.6. It is then measured into vials, about 1 to 2 ml. each, and lyophilized according to procedures described in Falosdorf, "Freezing-Drying," Reinhold, (1949) and Harris "Biological Applications of Freezing and Drying," Academic Press, New York (1954).

In use, the above-described novel composition may be reconstituted with an aqueous solvent such as deionized water and then incubated with a plasma of unknown coagulation. Reconstitution is effected at a ratio of about

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2 parts of the reconstituted novel composition to about 1 part of the unknown plasma and the time required for the formation of a clot at a temperature range of about 37° C. is observed.

The novel composition is designed to be used in such well known prothrombin time tests such as the Kato modification of the Quick test, the Link-Shapiro modification of the Quick test and the like. It can also be employed advantageously in the P—P test and its modification.

The following examples are included in order further to illustrate the invention.

EXAMPLE 1

Preparation of reagent

(1) Brain tissue from freshly killed rabbits is dried and pulverized in the presence of excess acetone and combined with an equal weight of ground rabbit lung tissue which had first been dried by a freezing-drying technique. The combined rabbit tissue is then extracted with an aqueous solution at 45°–50° C. This aqueous extract constitutes the thromboplastin fraction having strong activity towards human plasma.

(2) Bovine plasma is treated with a 10% by weight solution of barium sulfate by gentle agitation of the mixture. The barium sulfate is removed by centrifugation. This filtrate constitutes that component which is the plasma having a high concentration of Factor V and fibrinogen.

(3) Approximately equal volumes of the aqueous rabbit tissue extract and adsorbed plasma are then added to an aqueous solution of 0.0125 M calcium chloride and 0.075 M sodium chloride. The mixture is measured into vials in a volume of about 1 to 2 ml. and freeze-dried according to the method of Harris referred to above.

EXAMPLE 2

In order to illustrate the stability of the claimed composition in determining coagulation factors the following test is included.

The composition obtained as described in Example 1 containing tissue thromboplastin of strong activity, calcium ion and adsorbed bovine plasma containing a high concentration of Factor V and fibrinogen is marked for identification as composition A. A commercially available thromboplastin-calcium mixture such as the Owren composition marketed under the trade name of Thrombotest by Nyegaard & Co. (see U.S. Patent No. 3,179,567), which is made up essentially of tissue thromboplastin of weak activity towards human plasma, calcium ion, Factor V and fibrinogen is marked for identification as composition B. These compositions are then used in the determination of clotting factors according to the method of Quick described in the American Journal of Medical

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Sciences, 190: 501, 1935 and the results obtained for the clotting time observed in each instance is tabulated below:

	Fresh patient's plasma, sec.	Same plasma 72 hours later, sec.	Change, sec.
Composition A.....	18.3	21.1	+2.8
Composition B.....	51.5	65.3	+14.2

From the foregoing results it is obvious that the claimed composition gives a more accurate and consistent result in the Quick test for determination of coagulation factors than the known compositions and that the claimed composition also compensates for any aging or environmental effects of the plasma as apparent from the fact that after 72 hours of storage the claimed composition still gives substantially the same clotting time.

It is 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 diagnostic composition for use in determining blood coagulation factors comprising a tissue thromboplastin extract of strong activity, said thromboplastin being prepared from rabbit brain tissue which has been previously dried and pulverized in the presence of acetone and combined with about an equal part by weight a freeze-dried rabbit lung tissue, the combined tissues then being extracted with an aqueous solvent at 45 to 50° C. to produce said thromboplastin extract; a source of calcium ion at a concentration of about 0.0125 M; salt at a concentration of about 0.075 M; and plasma containing a high concentration of Factor V and fibrinogen, said plasma being prepared by treating plasma with barium sulfate to absorb Factors VII, IX, X and prothrombin, said lyophilized diagnostic composition being buffered at a pH from about 7.2 to 7.6.

2. Process for determining blood plasma coagulation factor levels which comprises reconstituting the composition defined in claim 1 with an aqueous solvent, mixing the resulting suspension with a plasma of unknown coagulation characteristics in a ratio of about 2 parts of said composition with about 1 part of said plasma and observing the time required for the formation of fibrin.

References Cited by the Examiner

UNITED STATES PATENTS

3,179,567 4/1965 Owren ----- 167—84.5

JULIAN S. LEVITT, *Primary Examiner.*

SAM ROSEN, *Assistant Examiner.*