April 20, 1965 P. A. OWREN 3,179,567

REAGENT AND METHOD FOR DETERMINING THE COAGULABILITY OF BLOOD

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FIG. 1.

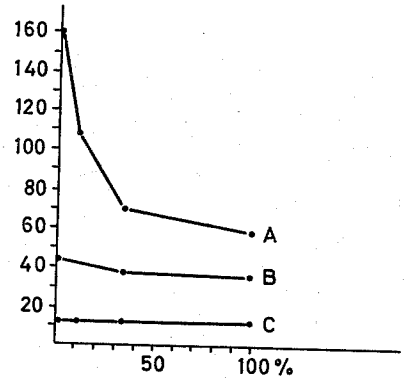


FIG. 2.

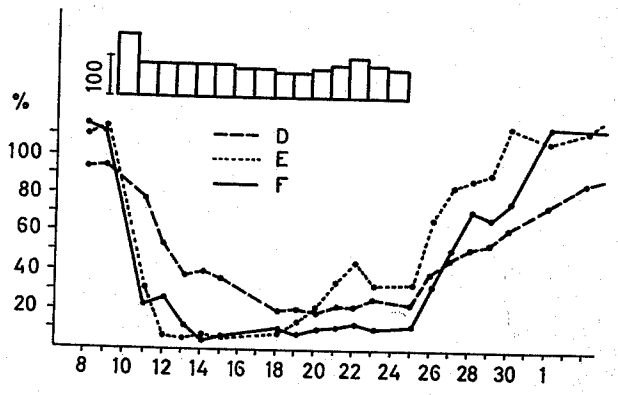
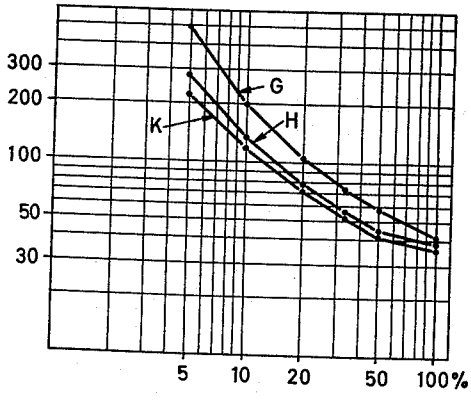


FIG. 3.



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REAGENT AND METHOD FOR DETERMINING THE COAGULABILITY OF BLOOD

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14 Claims. (Cl. 167—84.5)

Oral anticoagulants (dicumarol and dicumarol derivates, phenylindandione and similar substances) cause a reduction in the coagulability of the blood and are extensively used today in the treatment of thrombotic conditions. Prophylaxis against thrombosis in arteriosclerotic disease (coronary infarct, angina pectoris, cerebral and peripheral arterial thromboses) is being more and more generally adopted.

Effective anticoagulant treatment requires regular determinations of the coagulability of the blood. It is necessary to adjust the dosage to the individual case so as to avoid, on the one hand, excessive amounts which may result in bleeding complications, and, on the other, too small a dosage which will fail to be effective.

With previously described techniques for this control it has not been possible in each individual case to determine the coagulability of the blood with sufficient certainty so that these dosage difficulties are solved; there therefore exists a great need for methods which make accurate control possible.

By means of the reagent comprising the present invention, this problem is solved in a satisfactory way, as will appear from the following description of the invention.

In order to make it easier to understand the invention an account will be given of the action of anticoagulant agents.

Coagulation of the blood is effected by means of two partly separate coagulation systems: the external and the internal system. The peroral anticoagulant agents cause a decrease in the concentration of the following four coagulation factors in the blood: prothrombin, Stuart-factor, proconvertin and antihemophilia-B-factor. Of these factors, prothrombin and Stuart-factor are involved in both systems; proconvertin acts only in the external system; and antihemophilia-B-factor participates only in the internal system. This is shown in the following scheme:

Coagulation system of the blood

The "Internal" System (Cephalin Time)	The "External" System (Thromboplastin time)
Blood platelet factor 3 ("Cephalin"). Antihemophilia-A-factor (A.H.G.). Antihemophilia-B-factor (P.T.C.). Antihemophilia-C-factor (P.T.A.). Hageman-factor.	Tissue-thromboplastin. Proconvertin.
Staurt-factor. Prothrombin. Proaccelerin. Fibrinogen.	

Hitherto there has been no method available to make it possible to measure quantitatively the *total* effect of any reduction of the four specified factors on the whole coagulation process, i.e., on both the internal and external systems. The methods which have hitherto been used, namely: Quick's method and the so-called P-P-method, are sensitive only to the changes in the external coagulation system. Thus, applied to laboratory control of anticoagulant treatments they reflect the reduction of proconvertin, Stuart-factor and prothrombin; reduction of antihemophilia-B-factor is not registered at all by Quick's method and only to a very small extent by the P-P-method.

The accompanying FIGURE 1 shows the sensitivity of these three methods to decreasing quantities of antihemophilia-B-factor. "Concentration" of antihemophilia-B-factor relative to the other factors is given as a percentage of the normal value along the abscissa. The coagolation time in seconds (20 to 160 sec.) is plotted on the ordinate.

The study was made by mixing hemophilia-B-plasma and normal plasma in various proportions.

Curve A shows the sensitivity of the new method to diminishing quantities of antihemophilia-B-factor.

Curve B shows the sensitivity of the P-P-method and curve C shows the sensitivity of Quick's method to diminishing quantities of antihemophilia-B-factor.

The explanation of the differences seen in the curves shown in the figure is that in the P-P-method and in Quick's method a very active tissue-thromboplastin is added to the blood or plasma to be examined.

In Quick's method an extract of rabbit brain is used and in the P-P-method an extract of human brain. These very active thromboplastins cause the coagulation to occur exclusively via the external coagulation system, since clotting in this system is so rapid that it is over long before the internal system can become fully activated. Thus the normal coagulation time of a plasma following addition of an active thromboplastin, as in Quick's method, is only 12–13 seconds, whereas the internal system, even after addition of optimal cephalin concentration, requires at least 50 seconds to produce the formation of thrombin which in turn coagulates fibrinogen. Addition of such active thromboplastins completely circumvents the internal system. With Quick's method the coagulation time (which is often called "the prothrombin time" or "the thromboplastin time") is consequently perfectly normal even when there is complete absence of antihemophilia-B-factor (cf. FIG. 1).

It should be pointed out that methods have been worked out for determining the activity of the internal coagulation system, using cephalin instead of thromboplastin. These methods can, depending on the details of the system, be used for determination of the various antihemophilic factors. However, methods of this type are never used for control of anticoagulant treatment for two principal reasons. First, such "cephalin times" are very sensitive to a number of incidental effects; secondly, they are entirely unaffected by changes in the external coagulation system, of which variations in proconvertin concentration may be most important.

The present invention provides a method which permits the practitioner to determine simultaneously the coagulability of the blood in both the external and the internal system with a single test. By means of the new reagent the external and the internal coagulation system are started simultaneously and in such manner that they proceed at approximately the same rate, with the result that the two systems exert fairly equal influences on the coagulation time of the system.

Previous methods for control of anticoagulant treatment

Two principal methods have, as mentioned, been used hitherto for control of anticoagulant treatment: (1) Quick's method; (2) The P-P-method.

(1) *Quick's method.*—[Quick, A. J.: Biol. Chem. J. 109, lxxiii (1935).]

Quick's method was originally introduced as a method for the determination of the prothrombin of the blood; the factors proconvertin, Stuart-factor and proaccelerin were at that time unknown. In this method calcium and the thromboplastin concentrations are kept constant.

The method has undergone minor changes. The latest detailed description is the following: Quick, A. J.: Hemorrhagic Diseases, Lea and Fibiger, Philadelphia, 1957, pp. 379–387.

Reagents:

(1) Thromboplastin suspension: Extract of acetone-dried rabbit brain.

(2) Calcium chloride solution 0.01 M.

Procedure: 0.1 ml. oxalated plasma is mixed with 0.1 ml. thromboplastin solution, placed in water-bath at 37° C. and recalcified with 0.1 ml. of 0.01 M calcium chloride solution, and the coagulation time or the "thromboplastin time" (also called "Quick's prothrombin time") is determined.

Commentary: The method is sensitive only to the external coagulation system (cf. the scheme in column 1). The internal coagulation system does not, as stated earlier, have an opportunity to influence the coagulation time.

Thromboplastin and calcium are the only factors which are kept constant in Quick's method. A change in any of the other factors of the external system: prothrombin, proconvertin, Stuart-factor, proaccelerin or fibrinogen, would also, however, be able to affect the registered Quick's "thromboplastin time." During anticoagulant treatment it is probable that proaccelerin and fibrinogen are relatively constant; hence any prolongation of Quick's "thromboplastin time" will be due to a reduction of proconvertin, Stuart-factor, and/or prothrombin. On the other hand the method is entirely insensitive to a reduction of antihemophilia-B-Factor. In hemophilia-B Quick's "thromboplastin time" is normal (see FIG. 1). Therefore, if this method is used for control of the dosage in anticoagulant treatment, there is a risk that hemorrhage will occur because of a particularly large reduction of antihemophilia-B-factor, since such a change would not be revealed with this method. By specific quantitative determination of the various factors involved we have found that antihemophilia-B-factor is often reduced more markedly than the other factors, as can be seen from FIGURE 2. These results are for phenylindanedione, but Dicumarol and Dicumarol derivates have essentially the same effect.

On the abscissa the numbers 8–30 indicate the dates in March 1957 when the tests were performed. The tests were concluded on April 2, 1957.

On the ordinate are given percentages (these are not absolute, 100% signifying normal "concentration") of prothrombin, proconvertin and antihemophilia-B-factor found in the blood on the dates shown by analyses conducted during this investigation.

Curve D shows the percentage of prothrombin found in the blood; curve E, the percentage of proconvertin; and curve F, the percentage of antihemophilia-B-factor.

As can be seen, the level of the three mentioned factors was approximately 100% at the beginning of the tests. On March 9, 1957, the anticoagulant agent phenylindandione was begun in daily doses, the quantity of which can be seen in the area at the top of the figure. On the left of this area the vertical line represents 100 mg. phenylindandione. The quantity of each day's dose appears at the vertical line drawn from the date in question on the abscissa. On March 25 the administration of the anticoagulant agent was stopped and the percentage content of the factors then rose quickly.

The fact that Quick's method is not sensitive to reduction of antihemophilia-B-factor is thought to be one of the causes of the relatively large frequency of hemorrhagic complications when Quick's method is used for control of anticoagulant treatment.

Among other defects of this method may be mentioned the facts that it is insensitive to variations of all three of the factors in the range 50 to 100% of normal, that the calcium concentration employed is not optimal if the hematocrit value of the blood is abnormal, and that the "thromboplastin time" is affected by the proaccelerin concentration in the plasma examined. Proaccelerin is unstable and is inactivated during storage of oxalated plasma; for this reason the method requires a relatively fresh blood sample.

(2) *The P-P-method (prothrombin-proconvertin method).*—Owren, P.A.: The Coagulation of Blood, Acta Med. Scand. 128 (suppl. 194), p. 327 (1947); Owren, P.A.: Scand. J. Clin. Lab. Invest. 1, 81–83 (1949); Owren, P.A. and Aas, K.: Scand. J. Clin. Lab. Invest. 3, 201–208 (1951).

Reagents:

(1) Thromboplastin suspension: Aqueous extract of human brain.

(2) Adsorbed bovine plasma.

(3) Calcium chloride solution of optimal concentration (normally 0.025 M).

Procedure: 0.2 ml. adsorbed bovine plasma is mixed with 0.2 ml. thromboplastin suspension and 0.2 ml. of a 1:10-dilution of the plasma to be investigated; the mixture is placed in water bath at 37° C. The coagulation time (P–P time) is recorded after recalcification with 0.2 ml. calcium chloride solution.

Commentary: As in the case of Quick's method, the P–P-method is a determination of the "thromboplastin time," i.e. it is primarily a method for estimating the activity of the external coagulation system. The chief differences between the P–P-method and Quick's method are the following:

(1) A new reagent, adsorbed bovine plasma, is introduced in order to stabilize the concentration of proaccelerin and fibrinogen. The measurement is therefore independent of the concentration of these factors in the test plasma. Since the factors which are reduced during anticoagulant treatment are relatively stable during storage of blood and plasma, the method may be used on stored samples.

(2) A 10% dilution of the test plasma is used. This increases the sensitivity of the method, especially in the range 50–100%. Dilution of the test plasma also has the advantage that the risk of inoptimal calcium concentration with varying hematocrit values is far less than in Quick's method. The diluting also eliminates the effect of small amounts of heparin in the plasma to be examined.

As in the case of Quick's method, the P–P-method is sensitive to reductions of prothrombin, Stuart-factor and proconvertin. [Gonyea, L.M., Hjort, P., Owren, P.A.: The Journal of Laboratory & Clinical Medicine, 48, 624–633 (1956).]

The thromboplastin used in the P–P-method (aqueous extract of human brain) exerts, in addition to its thromboplastin effect, a cephalin effect on the internal coagulation system. This effect is, however, not optimal. As a result of this cephalin effect, and of the dilution of the test plasma, it can be shown that the P–P-method possesses a certain, though exceedingly small, sensitivity to excessive reductions of antihemophilia-B-factor, if the external coagulation system is greatly impaired following anticoagulant treatment (see FIG. 1). [Waaler, B. A.: Scand. J. Clin. Lab. Invest. 9, 322–330 (1957).] From the point of view of clinical practice this sensitivity of the P–P-method to reductions of antihemophilia-B-factor is far too small to permit it to provide certain protection against hemorrhagic complications due to severe depression of antihemophilia-B-factor.

From time to time other modifications of Quick's meth-

od have been suggested, such, for instance, as employment of other thromboplastin preparations—from rabbit lungs and human brain, some in acetone-dried form and others in frozen-dried form. Dilutions of the plasma to be investigated have also been used in order to increase the sensitivity of the method, especially in the range 50–100%. Variation of the calcium concentration relative to the hematocrit value of the blood have also been suggested in order to secure optimal recalcification. However, none of these modifications has altered the principle of the method, and none has involved any change in the method's defective sensitivity to reduction of anti-hemophilia-B-factor.

The reagents for the P–P-method—the adsorbed bovine plasma and the thromboplastin suspension—have been produced in frozen-dried form. However, this change has of course not altered the defective sensitivity of the method to reduction of antihemophilia-B-factor.

Method for control of the coagulability of the blood during anticoagulant treatment

From the foregoing it will be seen that none of the hitherto available aids has been satisfactory for the determination of the coagulation activity of the blood in order to control anticoagulant treatment.

The following account describes the invention: a preparation which permits satisfactory determination of blood coagulability as a control of this treatment.

This procedure is based on an entirely new principle, namely: that a simultaneous and combined determination is made of the activity in the internal and in the external coagulation system. Hitherto we have not had any method for such combined determination; the two coagulation systems have always been investigated separately.

The method is based on a reagent which initiates both coagulation systems simultaneously and in such a way that they both require approximately the same amount of time. To achieve this situation the internal coagulation system has been given optimal conditions for maximum speed by inclusion of "cephalin" from human brains or any other cephalin preparation which has the same activity, in the reagent in optimal concentration. The internal coagulation system alone will produce complete coagulation under these conditions in about 50 seconds.

At the same time the reagent contains a thromboplastin which starts the external coagulation system, but this thromboplastin is of such a nature that its maximal activity is low as compared with human brain thromboplastin and rabbit brain thromboplastin. As a result, the speed of the external coagulation system is considerably reduced as compared to the normal speed obtained with active thromboplastin preparations of the type used for Quick's method and the P–P-method. The thromboplastin used in this method should give a thromboplastin time with normal human plasma of 35–50 seconds, as tested by Quick's method.

Theoretically a weak thromboplastin could be produced by dilution of an active thromboplastin (of the type used for Quick's method and the P-P-method), but such a procedure results in a labile and practically useless reagent because small changes in its activity give considerable oscillations in the registered times. The reagent therefore employs a thromboplastin which has a stable and low activity in optimal concentration and which has a relatively wide range of optimal concentrations.

As starting material for production of such a thromboplastin, lung or brain of certain species of animals (bovine, horse, swine, sheep, dog) are used. The thromboplastin produced is species specific and reacts only slowly with human proconvertin, as revealed by the thromboplastin time of 35–50 seconds as compared with the thromboplastin time of 13–15 seconds of human brain thromboplastin.

The combination in one reagent of a thromboplastin having such weak activity toward human plasma and of cephalin, both in the concentrations of maximal activity, has been found to result in the internal and the external coagulation systems' having approximately equal influence on the coagulation time. This method is therefore sensitive to reductions both of antihemophilia-B-factor in the internal system and of proconvertin in the external system, in addition to Stuart-factor and prothrombin, which participate in both systems.

FIG. 1 shows the marked sensitivity of this method to large reductions of antihemophilia-B-factor, in contrast to the very defective sensitivity of Quick's method and the very slight sensitivity of the P-P-method.

It follows from this that the principal drawbacks of the methods and reagents hitherto employed (Quick's method, the P-P-method) have in all essential respects been eliminated.

The invention is, further, based on the concept that the coagulation times determined should be independent of changes in all the coagulation factors which are not affected by antocoagulant treatment. This is accomplished by the inclusion in the reagent of all these factors in high concentrations (actual concentrations specified below). For this purpose a specially adsorbed plasma (bovine, horse, swine, sheep, dog) prepared similar to that as for the P-P-method, is used. This adsorbed animal plasma contains high concentrations (minimal 90%—relative to human plasma) of antihemophilia-A-factor, antihemophilia-C-factor and Hageman-factor (of the internal system), and of proaccelerin and fabrinogen (which participate in both systems). It is, further, prepared in such a way that it does not contain perceptible amounts (as determined by the methods specified below) of proconvertin, antihemophilia-B-factor, Stuart-factor or prothrombin—precisely the four factors which are reduced during anticoagulant treatment.

As a result, the reagent is specific for and sensitive to all the four factors which are reduced during anticoagulant treatment, and is at the same time uninfluenced by variations in the other coagulation factors. The coagulation times which are registered by this method therefore provide an adequate index of the total effect which the anticoagulant agents have on the coagulation of the blood. The decisive difference from earlier methods is, as stated, the sensitivity of the new method to reduction of the concentration of antihemophilia-B-factor.

Composition of the reagent

The new reagent thus contains the following four components:

(1) *Thromboplastin.*—Every type of thromboplastin may be used, provided it fulfills the following requirements: (*a*) The thromboplastin suspension is stable on storage at 20° to 25° C. for at least 24 hours. (*b*) The optimal dilution of the thromboplastin suspension, which produces the maximal activity for the preparation as tested on normal human plasma, gives a thromboplastin time between 30 and 50 seconds as tested by the method of Quick. This thromboplastin time provides the desired balance between the internal and the external clotting systems.

Thromboplastin preparations having the specified activity have been prepared as follows:

(1) Extracts from bovine, horse, dog, swine and sheep brain. [Method—Owren, P. A.: Scand. J. Clin. Lab Invest. 1, 81 (1949).]

(2) Extracts from bovine, horse, swine, dog and sheep lungs. [Method—Ware, A. G. and Seegers, W. H.: Am. J. Clin. Path, 19,471 (1949).]

Examples of thromboplastin times produced by the various extracts are given in the table.

The thromboplastin times of normal plasma with different thromboplastins

Thromboplastin species:	Thromboplastin time of human plasma (seconds)
Human brain	13.2
Human lung	12.8
Rabbit brain	12.0
Rabbit lung	13.2
Ox brain	33.5
Ox lung	31.0
Horse brain	30.5
Horse lung	31.0
Swine brain	36.2
Swine lung	35.3
Dog brain	33.5
Dog lung	32.6
Sheep brain	38.2
Sheep lung	34.3

Human brain thermoplastin, giving a thromboplastin time of 13–15 seconds, and rabbit brain thromboplastin, producing a thromboplastin time of 12–13 seconds, cannot be used.

The bovine brain thromboplastin is most useful. A detailed technique of its preparation is given below. The optimal concentration of this thromboplastin giving maximal activity contains 1.3–1.7 g. of brain substance, weighed in the dried state, per 100 ml. of solution.

(2) *Cephalin.*—All "cephalin" preparations may be used if they fulfill the following requirements: (*a*) The cephalin suspension must be stable for at least 24 hours at 20° to 25° C. (*b*) The cephalin time produced with normal non-activated (prepared by silicon technique) platelet-poor [centrifuged for 30 minutes at 3000 r.p.m. (1,800 g. at 4° C.)] human plasma must be shorter than 55 seconds. [Technique as described from this laboratory by B. A. Waaler: Scand. J. of Clin. & Lab. Investigation 9, 322 (1957).] (*c*) The cephalin time of non-activated platelet-poor plasma from a patient congenitally deficient in proconvertin (factor VII) is the same as for normal plasma, below 55 seconds. This test is applied to exclude that part of the activity produced on normal plasma which is caused by contamination of the cephalin preparation with thromboplastic activity.

The following cephalin preparations have been tested and found suitable: (*a*) Crude cephalin prepared from human brain. (*b*) Cephalin prepared from soya beans. [One such preparaiton is available commercially: Asolectin (Associated Concentrates Inc., 57—01 32nd Avenue, Woodside, New York).] (*c*) Phosphatidylethanolamine prepared by the method of O'Brien. [O'Brien, J. R.: J. Clin. Path. 9, 47 (1956).]

The coagulation active principles in the cephalin preparations are phosphatidylethanolamine, phosphatidylserine, and possibly other similar substances. There are great difficulties in obtaining large quantities of individual phospholipids. The two first preparations are completely satisfactory for this reagent, however. Detailed techniques for the preparation of crude human brain cephalin is given below. The optimal concentration of crude cephalin in the final reagent, is 0.001–0.01 g. per 100 ml. of reagent and of asolectin 0.010–0.015 g. of dried substance per 100 ml. of reagent.

(3) *Adsorbed plasma.*—Any plasma with the following characteristics may be used:

(A) It must be completely free of the following 4 clotting factors: prothrombin (factor II), proconverten (factor VII), Stuart-Prower-factor (factor X) and antihemophilic-B-factor (factor IX), as tested by the following methods—

(1) Thromboplastin time (Quick's method, see above): more than 24 hours.

(2) The Russell viper venom cephalin time [method—Hjort, P., Rapaport, S. I., Owren, P. A.: The Journal of Laboratory & Clinical Medicine 46, 89 (1955)]: More than 3 hours.

(3) Antihemophilia-B-factor [method—Stapp, W. F.: Scand. J. Clin. Lab. Invest. 10, 169 (1958)]: No measurable activity.

(B) It must contain a concentration (relative to human plasma) of the following activities of at least 90 percent.

(1) Proaccelerin (factor V) activity [method—Owren, P. A.: The Coagulation of Blood (1947)].

(2) Antihemophilia-A-factor activity [method—Waaler, B. A.: Scand. J. Clin. Lab. Investigation ii (1959)].

(C) It must not contain measurable amounts of "activation product" (as tested by the method of Waaler, B. A.: Scand. J. Clin. Lab. Investigation Suppl. 11, 1959, page 32).

Adsorbed plasma fulfilling the requirements given above have been prepared from bovine, horse, dog, sheep and human plasma. Bovine plasma is preferred because of higher concentrations and greater stability of proaccelerin and antihemophilia-A-factor on storage.

Technique of preparation is identical for all species. Details of preparation from bovine plasma are given below.

(4) *Calcium chloride.*—A one molar aqueous stock solution was prepared by dissolving 111.1 g. of anhydrous $CaCl_2$ (analytical grade) in 1000 ml. of distilled water.

Technique of preparation is given by examples as follows:

(1) Preparation of bovine brain thromboplastin [method similar to Owren, P. A.: Scand. J. Lab. Clin. Invest. 1, 81 (1949)].

Bovine brain obtained shortly after death was stripped of the pia and blood vessels and rinsed in running tap water. The cerebellum and the medulla were discarded. The brain was cut in small pieces and again rinsed in running tap water. To 250 g. of brain tissue were added 1000 ml. of saline (45° C.), and the mixture was blended for 4 minutes in a "mix master" ("Ato-Mix" blender and disintegrator). After standing for 1 hour the mixture was centrifuged for 30 minutes at 2500 r.p.m. and the supernatant collected. The precipitate was discarded. The pH of the supernatant was adjusted to 7.35 with 0.5 normal NaOH, and 1/10 volume of veronal buffer (pH 7.35 and ionic strength 0.154) was added. This buffer was made by mixing 11.75 g. sodium diethyl barbiturate, 14.67 g. sodium chloride, 430 ml. 0.1 normal HCl and distilled water to 2,000 ml. The activity of a sample of the thromboplastin solution was tested in serial dilutions in physiological saline by Quick's method (0.20 ml. oxalated normal human plasma, 0.20 ml. thromboplastin solution, 0.20 ml. calcium chloride solution, 20 mm.) The dilution 1:1 gave maximal activity of 34.5 seconds. The thromboplastin solution was consequently diluted with an equal volume of physiological saline solution. The final solution contained 1.4 g. of dried brain substance per 100 ml. of suspension.

(2) Preparation of human brain cephalin [method—Hjort, P., Rapaport, S. I., and Owren, P. A.: J. Lab. Clin. Med. 46, 89 (1955)].

The crude "cephalin" suspension is the acetone-insoluble, ether-soluble fraction of human brain. It was prepared as follows: Four hundred grams of human brain were washed free of blood and membranes and ground with 300 ml. of acetone. (Merck & Company, Inc.) After centrifugation, the acetone was discarded and the residue re-extracted with the same volume of acetone a total of six times. The residue was then extracted with 1,800 ml. of ether overnight at room temperature. The ether was siphoned off and evaporated to dryness by suction at 35° C. The resulting residue was washed twice for 15 minutes with 900 ml. of acetone and then redissolved in 200 ml. of ether. The ether was again evaporated to dryness and the suction continued for 75 minutes. About 9.3 g. of a waxy cream-colored to

brown residue were suspended in 200 ml. of Veronal buffer. This was centrifuged for 20 minutes at 1,700 r.p.m. and again for 10 minutes at 2,500 r.p.m. The sediment was discarded. The supernatant, which contained 2.8 g. percent of crude cephalin in a milky white suspension, was frozen in small quantities at −20° C.

Before use a sample of the melted suspension was tested in serial dilutions with physiological saline for determining the optimal dilution giving maximal activity in the cephalin time test. The shortest cephalin time with normal human plasma, being 52 seconds, was effected by a dilution of 1:200. The cephalin time of proconvertin deficient plasma was 51 seconds. The cephalin suspension was diluted 1:200 in the thromboplastin suspension to a concentration of 0.014 g. percent. The final reagent before freeze-drying contained 2/5 of this thromboplastin suspension and consequently a concentration of 0.0056 g. percent "cephalin."

(3) Preparation of adsorbed bovine plasma:

(*a*) Collection of blood. One hundred ml. of 2.5 percent (w./v.) potassium oxalate monohydrate solution are added into perfectly siliconized ("Siloxan 300," Uddeholms Aktiebolag, Uddeholm, Sweden) one liter glass bottles. The bottles are kept in packed ice, and filled with 900 ml. of bovine blood from the jugular vein by puncture with a coarse siliconized needle ("Monocote," Armour Laboratories, Eastbourne, England). All the following steps are performed at low temperature (between +2° C. and 4° C.) and by the use of siliconized equipment.

(*b*) The bottles are centrifuged for 30 minutes at 2,500 r.p.m. (1,700 g.).

(*c*) The plasma is siphoned off into beakers.

(*d*) The plasma is adsorbed with analytical grade $BaSO_4$ (Baker) in an amount of 75 mg. per ml. of plasma for 15 minutes and by slow stirring with a glass rod.

(*e*) The plasma is centrifuged for 30 minutes at 2,500 r.p.m. (1,700 g.) and the sediment discarded.

(*f*) A second adsorption with $BaSO_4$ is performed in the same way.

(*g*) A sample of the adsorbed plasma is tested with the Russel viper venom cephalin method. If any visible clot appears within 2 hours, a third adsorption is performed.

(*h*) The adsorbed plasma is passed through a 20 percent asbestos paper filter pad (Carlson Ltd., London), filter diameter 15 cm., CO_2 pressure 1 kg. per square cm., filter changed every 500 ml.

(*i*) The plasma is dialyzed for 12 hours against ten times the plasma volume of 0.85 percent saline solution, the saline being changed three times (Cellulose Casings, Visking Company, Chicago).

(*j*) The dialyzed plasma is adjusted to pH 7.35 with N/2 HCl (Radiometer, Copenhagen, Denmark). Accuracy ±0.05.

(*k*) The plasma is tested with the test methods referred to above. If the plasma does not fulfill the requirements stated, it is discarded.

(4) The preparation of calcium chloride solution of 12.5 milli-molar:

A 50 millimolar solution of calcium chloride is prepared by first mixing 50 ml. of the calcium chloride stock solution of 1 molar and 950 ml. of distilled water. One volume of this solution and 3 volumes of physiological saline solution are then mixed (this technique provides correct ionic strength).

Preparation of the reagent:

Equal volumes of adsorbed bovine plasma and the thromboplastin cephalin suspension are mixed. To the mixture is added 1/5 its volume of a calcium chloride solution of 12.5 millimolar.

Freeze-drying

The following example is illustrative of the procedure that may be used to prepare the freeze-dried product.

The freeze-drying of the mixture mentioned above is performed in ampules of Jenaglass of 30 ml. volume. The ampules are of the usual type, capable of being sealed so as to preserve its contents.

10 ml. of said mixture are poured carefully into the ampule. The ampule with its contents is first cooled to a temperature of about −70° C. During the cooling to said temperature, the ampule is situated in a slightly slanting position, approximately 4 to 6 degrees relative to the horizontal, and further the ampule is brought to rotate during said cooling. The cooling period is about ½ to 1 hour.

The freeze-drying is then carried out under high vacuum and with supplying of heat to the ampule, for example so that the ampule is in contact with suitable elements, to 60–70° C., so long as ice is still present in the mixture; and then the temperature is lowered to about 40° C. during the final drying step.

The product is treated under vacuum until the water content is below 0.5%. The ampule is then sealed, preferably by closing the ampule opening by means of fusing under vacuum.

Comments

The preparation of cephalin, thromboplastin and calcium solutions follows previously known principles. In the preparation of adsorbed plasma the following points are stressed:

(1) Careful silicone technique has to be applied in order to prevent the activation of clotting factors by contact with glass. Such activation includes a reaction comprising the antihemophilia-C-factor and the Hageman-factor producing an "activation product" which shortens the cephalin time. The "activation product" becomes slowly inactivated. A reagent containing "activation product," therefore, is unstable. The significance of this fact has not previously been understood.

(2) The adsorption procedure has been carefully standardized in order to obtain complete removal of the four factors mentioned. A reagent containing adsorbed plasma which does not clot on addition of thromboplastin and calcium has not previously been described.

(3) Careful centrifuging and filtering for complete removal of the suspended barium sulphate is essential. Residual particles of barium sulphate in the reagent with clotting factors adsorbed on the surface of the particle will produce thrombin formation on recalcification followed by the formation of fibrin.

(4) As a new principle in the preparation of this reagent, dialysis is introduced in order to obtain:

(*a*) Removal of the plasma's carbonic acid-bicarbonate buffer system. This is necessary for exact adjustment and stabilization of pH at 7.35 in the finished dried preparation. If CO_2 remains, it will escape during freeze-drying followed by rising pH.

(*b*) Removal of oxalate. This is necessary for (1) stability of the proaccelerin, (2) to prevent precipitation of calcium oxalate with consequent adsorption of coagulation factors during performance of the test, and (3) to permit use of citrate plasma for the determination.

(5) After dialysis the pH is adjusted to pH 7.35. This has significance for the stability of the reagent and for the solubility of fibrinogen on reconstruction.

(6) The final "all-in-one" reagent is freeze-dried in ampules for distribution.

(7) Stability of the freeze-dried reagent. The reagent shows unchanged activity after storage of the ampules at 47° C. for 30 days.

(8) The freeze-dried reagent is reconstituted by addition of distilled water or calcium chloride solution to the ampule for the capillary and venous blood method, respectively.

(9) Stability of the reagent in solution.

The reagent in solution before freeze-drying as well as after reconstitution of the dried reagent shows unchanged reactivity even after storage at room temperature for 12 hours. This stability is obtained, inter alia, by the total

elimination which has been achieved of the above-mentioned four coagulation factors. It has formerly been possible to mix thromboplastin and calcium into one reagent, but a stable reagent has not been previously possible after recalcification of an adsorbed plasma with or without thromboplastin.

Procedure for use

When using the preparation for determining the coagulability of the blood, the following procedure is employed:

After reconstitution of the reagent in distilled water or calcium chloride solution (see below), 0.5 ml. is placed by means of a pipette in a very small test tube (diameter 8–9 mm., length 60 mm.). A measured quantity of the plasma or blood to be examined (see below) is added and the coagulation time determined with a stop-watch. The recorded time is an expression of the effect of the anticoagulant treatment on the coagulation process. The test is performed in a water-bath at 37° C.

The registered coagulation time is converted to relative activity, stated in percentage, using a correlation curve. Examples of correlation curves are given in FIGURE 3. These correlation curves show the ratio between recorded coagulation times in two systems and the coagulation activity expressed in percentage of the normal.

The concentration of normal plasma in percentage is plotted on the logarithmic scale of the abscissa and the coagulation times in seconds (30–300 sec.) are plotted on the logarithmic scale of the ordinate. Curve K shows the method used for capillary blood, 0.10 ml. volume. Curve H shows the method used for citrate blood, 0.10 ml. volume, and for citrate plasma in dilution: 3 parts of plasma to 2 parts of saline, and 0.10 ml. volume.

Curve C shows an alternative method used for citrate plasma in dilution: 1 volume of plasma to 4 volumes of saline, and 0.20 ml. of the dilution as test volume.

The method can be used both for capillary blood (whole blood), which contains no added anticoagulant, and for citrate blood or citrate plasma. The reagent is provided with optimal concentration for capillary blood and cannot be used directly for citrate blood or citrate plasma, since for such samples the calcium concentration is inoptimal.

For citrate blood and citrate plasma, therefore, the dried reagent has to be reconstituted with 3.2 millimols calcium chloride solution which compensates for the amount of citrate in the test sample.

(1) Capillary blood:

A skin incision which affords lively bleeding should be made in the finger tip or the ear lobe. 0.10 ml. of whole blood is then filled into a pipette in the usual way, and this is at once mixed with the 0.5 ml. reagent which has previously been placed in the water-bath at 37° C. The coagulation time is determined.

(2) Citrate blood:

Venous blood is used mixed with 3.13 percent (w./v.) sodium citrate dihydrate solution in the ratio 1 part sodium citrate solution to 9 parts of blood. Example: 0.2 ml. sodium citrate solution is drawn into a 2 ml. syringe, and the syringe is then filled with blood by venipuncture to a volume of 2 ml. The sample is transferred to a small test tube and 0.10 ml. citrate blood is removed with a pipette and mixed with 0.5 ml. reagent, and the coagulation time is determined in the manner described above.

(3) Citrate plasma:

Citrate blood is prepared in the manner described in paragraph (2). After centrifuging, 0.6 ml. citrate plasma is measured off into a small test tube and 0.4 ml. physiological salt solution added. After mixing, 0.10 ml. of the diluted plasma is removed by pipette and at once mixed with .05 ml. of the reagent. The coagulation time is determined as indicated above.

The new "all-in-one" reagent constitutes a new creation in that it makes it possible to perform a combined determination of the coagulation activity of both the internal and the external coagulation systems.

The preparation of the reagent is based on principles which have not previously been employed. The reagent has a number of advantages when compared with earlier reagents: (1) It is very stable at room temperature, thus simplifying distribution and storage. (2) It withstands heating up to 47° C. for a minimum of 30 days, and can therefore be used in tropical regions. (3) The requirement for only one reagent makes the procedure simpler and the performance of the test faster than was the case for earlier methods. (4) It is the only method so far devised which provides an adequate index of the coagulation disturbance produced by oral anticoagulant agents. In contrast to earlier methods this one is sensitive to reduction of antihemophilia-B-factor. (5) As a consequence of the above qualities, this method gives far greater protection against hemorrhagic complications than other methods. (6) The method is time-saving. (7) Because of the stability of the reagent in reconstituted form it affords reliable and reproducible results.

I claim:

1. A process which comprises

(I) admixing

(*a*) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep,

(*b*) free cephalin from human brain and

(*c*) adsorbed plasma, said plasma being selected from the group consisting of bovine, horse, swine, dog and sheep plasma, which plasma has a high concentration of all factors which are not reduced during anticoagulant treatment, and

(II) alding to the resulting admixture calcium chloride in an amount to yield optimal concentration for coagulation after addition of test plasma.

2. A combination of thromboplastin from an animal organ and free cephalin in a single composition, the thromboplastin being a member selected from the group consisting of brain thromboplastin and lug thromboplastin from at least one of bovine, swine, dog and sheep, the thromboplastin being stable on storage at from about 20° to about 25° C. for at least 24 hours and having a maximum activity on normal blood plasma to yield a thromboplastin time between 30 and 50 seconds, and the cephalin having a high reactivity toward the internal clotting system of human plasma to yield a cephalin time of less than 55 seconds.

3. A composition for determining the coagulability of blood for control of treatment with anticoagulant agents, the composition comprising as essential constituents the combination consisting essentially of (*a*) animal organ thromboplastin which has a weak but constant reactivity toward the external clotting system of human blood plasma, the thromboplastin being a member selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, and (*b*) free cephalin having a high reactivity toward the internal clotting system of human plasma, the correlated proportions of the thromboplastin and the free cephalin assuring approximately equal influence by both internal and external coagulation systems on coagulation time.

4. A composition for determining the coagulability of blood for control of treatment with anticoagulant agents, the composition comprising:

(*a*) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(*b*) adsorbed plasma, from which plasma prothrombin, Stuart-factor, proconvertin and antihemophilia-

B-factor have been removed, said plasma being a member selected from the group consisting of bovine, horse, swine, dog and sheep plasma, and

(c) free cephalin produced from human brain, said cephalin having a high reactivity toward the internal clotting system of human plasma,

the influence of both internal and external coagulation systems on coagulation time being maintained approximately equal by the relative concentrations of the thromboplastin and the free cephalin.

5. A blood coagulability-determining composition consisting essentially of

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(b) adsorbed plasma, from which plasma prothrombin, Stuart-factor, proconvertin and antihemophilia-B-factor have been removed, said plasma being a member selected from the group consisting of bovine, horse, swine, dog and sheep plasma, and

(c) a free cephalin preparation having a high reactivity toward the internal clotting system of human plasma,

the influence of both internal and external coagulation systems on coagulation time being maintained approximately equal by the relative concentrations of the thromboplastin and the free cephalin.

6. A blood coagulability-determining composition comprising:

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma, and

(b) free cephalin from human brain and having a high reactivity toward the internal clotting system of human plasma,

the concentrations of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal the influence on coagulation time of both internal and external clotting systems.

7. A blood-coagulability-determining composition comprising:

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma, and

(b) a free cephalin preparation from soya beans and having a high reactivity toward the internal clotting system of human plasma,

the concentrations of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal the influence on coagulation time of both internal and external clotting systems.

8. A blood coagulability-determining composition comprising:

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(b) free cephalin having a high reactivity toward the internal clotting system of human plasma, and

(c) calcium chloride in an amount which gives optimal concentration for coagulation after reconstitution and addition of test plasma,

the concentration of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal the influence on coagulation time of both internal and external clotting systems.

9. A blood coagulability-determining composition comprising:

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(b) free cephalin from human brain and having a high reactivity toward the internal clotting system of human plasma, and

(c) calcium chloride in an amount which gives optimal concentration for coagulation after reconstitution and addition of test plasma,

the concentrations of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal to the influence on coagulation time of both internal and external systems.

10. A blood coagulability-determining composition comprising:

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(b) a free cephalin preparation from soya beans and having a high reactivity toward the internal clotting system of human plasma, and

(c) calcium chloride in an amount which gives opimal concentration for coagulation after reconstitution and addition of test plasma,

the concentrations of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal the influence on coagulation time of both internal and external clotting systems.

11. A blood coagulability-determining composition comprising:

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(b) adsorbed plasma, from which plasma prothrombin, Stuart-factor, proconvertin and antihemophilia-B-factor have been removed, said plasma being a member selected from the group consisting of bovine, horse, swine, dog and sheep plasma,

(c) free cephalin from human brain, and

(d) calcium chloride in an amount which gives optimal concentration for coagulation after reconstitution and addition of test plasma,

the concentrations of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal the influence on coagulation time of both internal and external clotting systems.

12. A blood coagulability-determining composition comprising:

(a) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(b) adsorbed plasma, from which plasma prothrombin, Stuart-factor, proconvertin and antihemophilia-B-factor have been removed, said plasma being a member selected from the group consisting of bovine, horse, swine, dog and sheep plasma,

(c) a free cephalin preparation from soya beans and having a high reactivity toward the internal clotting system of human plasma, and

(*d*) calcium chloride in an amount which gives optimal concentration for coagulation after reconstitution and addition of test plasma,

the concentrations of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal the influence on coagulation time of both internal and external clotting systems.

13. A blood coagulability-determining composition comprising:

(*a*) thromboplastin selected from the group consisting of brain thromboplastin and lung thromboplastin from at least one of bovine, horse, swine, dog and sheep, the thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma,

(*b*) adsorbed plasma free from prothrombin, Stuart-factor, proconvertin and antihemophilia-B-factor and containing a high concentration of all coagulation factors which are not affected by peroral anticoagulant agents, said plasma being selected from the group consisting of bovine, horse, swine, dog and sheep plasma,

(*c*) free cephalin having a high reactivity toward the internal clotting system of human plasma, and

(*d*) calcium chloride in an amount which gives optimal concentration for coagulation after reconstitution and addition of test plasma,

the concentrations of said thromboplastin and said free cephalin being correlated in the composition to maintain approximately equal the influence on coagulation time of both internal and external clotting systems.

14. A blood coagulability-determining composition comprising:

(*a*) bovine thromboplastin having a weak but constant reactivity toward the external clotting system of human blood plasma and

(*b*) free cephalin having a high reactivity toward the internal clotting system of human plasma.

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