PLASMINOGEN ASSAY SYSTEM


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ABSTRACT OF THE DISCLOSURE

Plasminogen in blood plasma is quantitatively determined by measuring the diameter of the clear radial diffusion zone produced by the reaction of the blood plasma with known amounts of clotted fibrinogen and plasminogen suspended in a stable gel medium.

This is a continuation of application Ser. No. 764,628, filed Oct. 2, 1968, now abandoned.

This invention relates to a method for the determination of fibrinolytic activity and, more particularly, to a method for the quantitative determination of fibrinolytic activity in blood plasma and fractions thereof.

The clotting of blood is of profound biological significance. It involves a complex mechanism and depends upon the presence and activity of a number of plasma proteins. Of these proteins, fibrinogen is of primary importance since the fundamental feature of the clotting mechanism is the conversion of a liquid clot into a rigid insoluble fibrin clot. This transformation is normally brought about by the interaction of fibrinogen and thrombin. Thrombin is derived from its precursors, prothrombin. The transformation of prothrombin into thrombin generally requires the presence of calcium ions and a member of a group of activating agents known as thromboplastins.

The fibrin clot disintegrates over a period of time as a result of enzymic action. The enzyme involved in this action is referred to as fibrinolysin (or plasmin). This proteolytic enzyme is present in normal blood in the form of its inert precursor, proplasminogen (or plasminogen). The plasminogen can be activated or rendered proteolytic by the use of certain reagents, for example, cheloforin, streptokinase, urokinase and other such plasminogen activators. These activators cause the conversion of plasminogen into plasmin which attacks the coagulation proteins of the fibrin clot. Inhibitors normally present in the blood with plasminogen generally retard this reaction.

The term "fibrinolytic system" is defined herein as the process of disintegration of the fibrin clot and the term "fibrinolytic activity" is defined herein to include the above-described chemical and physical activities involved in this disintegration.

The known methods for the determination of fibrinolytic activity generally employ reaction in a so-called "wet system." For example, Astrup et al., Arch. Biochem. and Biophys., vol. 40, pp. 346—351 (1952), disclose a wet fibrin plate system for the determination of plasmin activity in blood.

It is an object of the present invention to provide a new and improved method for the determination of fibrinolytic activity in blood plasma and fractions thereof.

It is another object of the present invention to provide a novel method for the quantitation of the various components of the fibrinolytic system.

In accordance with the present invention a stable gel is prepared from the gel medium and the fibrinolytic system for the quantitative determination of fibrinolytic activity. A fibrinolytic component of the fibrinolytic system comprising clotted fibrinogen is homogeneously suspended in the gel and allowed to react with an unknown sample comprising blood plasma or a fraction thereof containing other essential components of the fibrinolytic system for a predetermined period of time to produce a clear radial diffusion zone of reaction products at the interface of the unknown sample and the gel. The diameter of the radial diffusion zone is directly proportional to the log of the fibrinolytic activity in the sample being tested. The fibrinolytic activity in an unknown sample is determined by comparison with control samples of known fibrinolytic activity.

The term "stable" gel is defined herein as a gel that is inert or chemically non-reactive with the components of the fibrinolytic system suspended in the gel.

In preparing the gel medium from about 10% to about 10.0% by weight of a gelling agent is dissolved in a heated buffer system and then mixed with a predetermined amount of a clotted fibrinogen component of the fibrinolytic system as hereinbefore stated. Generally, from about 10 mg. percent to about 2 gm. percent, and preferably about 130 mg. percent, of fibrinogen is employed in the heated buffer system. While still hot, this mixture is poured into a low-sided flat receptacle (hereinafter also referred to as a plate) in an amount appropriate for the size of the receptacle. The mixture is allowed to gel, and wells or cylindrical holes of approximately 1 to 10 mm. in diameter are punched or otherwise formed in the gel.

Known and unknown samples, respectively, comprising blood plasma or a fraction thereof, containing other essential components of the fibrinolytic system are added to the open wells in the gel by use of a calibratory pipette or similar device and the plate is incubated at about 20° to 60° C., and preferably at about 37° C., for a predetermined period of time. During this incubation, a fibrinolytic reaction occurs as the material in the wells diffuses outwardly into the gel medium and produces a clear radial diffusion zone of reaction product.

At the end of the incubation period, the gel is visually inspected, preferably with the assistance of a light magnifying view having a scale for measurement of the diameter of the radial diffusion zone. By comparing the diameters of the clear radial diffusion zones produced during the incubation period by the reaction of the fibrinolytic component in the gel with the fibrinolytic components in known and unknown samples, respectively, the fibrinolytic activity in the unknown sample can be readily determined.

In the preparation of the gel medium, any conventional gelling agent can be used, for example, gelatin, pectin, silicone gel, starch, polysaccharides from seaweed such as agar, algin and carrageenin, synthetic polymeric gelling agents such as the cross-linked polycrylamide disclosed in U.S. Pat. 3,046,201 and the like materials. The gelling agent preferably has the physical properties characterizing agar-agar, and as it is readily dispersible in water and capable of forming an essentially clear hydrogel of sufficient rigidity so that the receptacle or plate containing the gel can be inverted without danger of the gel failing out.

A purified agarose is the preferred gelling agent employed in the present invention. Agarose is the neutral galactose polymer which has been separated from the agaropeptin fraction of agar by any conventional method, for example, the methods described in U.S. Pat. 3,281,409, 3,355,127 and 3,362,884. This gelling agent is preferably used at a concentration of about 2.5% by weight of the gel medium.

The buffer system generally employed in the gel has a pH of from about 6.0 to about 8.5 and preferably from about 7.3. A preferred buffer comprises 0.154 M sodium chloride, 0.04 M imidazole and 0.02 M ethylenediaminetetraacetic. Other suitable buffers which can be used.
are, for example, tris(hydroxymethyl)aminomethane, phosphate and barbital buffers.

In order to prepare a plate containing the gel with clotted fibrinogen, suitable clotting can be produced by dipping the plate containing the solidified gel and fibrinogen in a thrombin solution for an appropriate length of time, for example, from about 0.1 to about 15 minutes. The gel containing the clotted fibrinogen is then ready for punching of the wells as hereinbefore stated.

The gel plate containing the clotted fibrinogen can be overlaid with a protective membrane, packaged by various means, and thereby made conveniently available for subsequent use in the determination of fibrinolytic activity by hospitals, laboratories and other agencies and persons having a need for a simplified, yet accurate, determination of fibrinolytic activity in blood plasma samples. The packages for these plates can be, for example, plastic film or metal foil bags, pouches and the like, preferably sterilized and sealed to prevent the admission of air, moisture, dirt and other contaminating materials. Suitable metal foil can be fabricated, for example, from aluminum and the like metals; suitable plastic film can be fabricated, for example, from vinyl chloride and vinylidene chloride polymers and copolymers, polyvinyl chloride, polyvinyl alcohol, polyethylene, polypyrrole, polyesters, cellulose acetate and propionate, cellulose triacetate, cellulose acetate butyrate, ethyl cellulose, fluorocarbons, acrylic plastics such as acrylates and methacrylates, and polystyres such as, for example, the polystyres formed by condensation reactions between ethylene glycol and terephthalic acid.

The clotted fibrin plates can also be packaged in combination with control samples, buffer materials, capillary tubes and other components for making the complete fibrinolytic assay.

According to two preferred aspects of the present invention, the fibrinolytic component in the gel media and the fibrinolytic activity to be determined in the unknown sample are as follows:

Plate I. Clotted fibrinogen is suspended in the gel and determinations are made for (a) total plasminogen, (b) available plasmin, (c) active plasmin and (d) fibrinolytic inhibitor in the unknown sample.

Plate II. Clotted fibrinogen and plasminogen are suspended in the gel and a determination is made for plasminogen activator in the unknown sample.

In Plate I, a highly purified fibrinogen is used as the fibrinolytic component in the gel.

In Plate II, the fibrinolytic component comprises fibrinogen and plasminogen. This component can be prepared by using a fibrinogen which is contaminated with a known amount of plasminogen or by using a highly purified fibrinogen as in Plate I to which a predetermined amount of plasminogen is added. When a highly purified fibrinogen is employed, it is preferred to add the plasminogen to a final concentration of from about 0.01 to about 20 units/ml and most preferably about one unit/ml. As used herein, one unit/ml of plasminogen is defined as the amount of plasminogen in pooled normal plasma.

The following examples will further illustrate the invention although the invention is not limited to these specific examples. All parts and percentages herein are by weight unless otherwise specified.

**EXAMPLE 1**

An agarose gel plate is prepared as follows:

**Preparation of reagents**

(A) Agarose gel plate buffer—0.154 M sodium chloride, 0.04 M imidazole and 0.02 M ethylenediaminetetra-acetate, pH 7.30.

(B) Glycine citrated saline solution—0.123 M sodium chloride, 0.020 M sodium citrate and 0.1 M glycine.

(C) Thrombin solution—Parke-Davis topical thrombin, bovine origin, 5000 units/vial, is diluted to 50 units/ml with cold distilled water, the pH is adjusted to 5.30 with cold 1.0 N acetic acid, the resultant precipitate is spun down in a refrigerated centrifuge and the supernate is decanted and lyophilized. The lyophilized product is reconstituted to its original volume with sterile, distilled water prior to use.

**Procedure**

A highly purified fibrinogen solution is prepared as follows:

Pool human plasma is frozen and then slowly thawed to 4°C. The cryoprecipitate is removed by centrifugation and dissolved in 1/30 the original plasma volume of glycine citrated saline. The pH of the solution is adjusted to 6.50 with 1.0 Normal acetic acid. Then 3.5 gm. percent of polyethylene glycol 4000 (mol. wt. ca. 4000) is slowly added to the above solution and precipitation is carried out with stirring for 15 minutes at room temperature. The resultant precipitate is spun out by centrifugation and dissolved in glycine citrated saline (1/30 the original plasma volume). The pH of the solution is adjusted to 6.88 with 1.0 Normal sodium hydroxide and the solution is then cooled to 9°C. Glycine is slowly added to the solution to make the final glycine concentration 1.8 M and the precipitate is carried out with stirring for 45 minutes at 5°C. The resultant precipitate is spun out by centrifugation and dissolved in normal physiologic saline to produce a concentration of approximately 2.5 gm. percent fibrinogen. The fibrinogen solution is then adsorbed three times with 5 gm. percent Darco G–60 charcoal. Each adsorption is carried out for thirty minutes at room temperature, the solid charcoal being removed after each process by centrifugation. The final solution is adjusted to 260 mg. percent fibrinogen by appropriate dilution with normal physiologic saline. The solution is then stored in the frozen state.

An agarose solution is prepared as follows: 2.5 gm. percent agarose is completely dissolved in the above-described agarose gel plate buffer.

A clotted fibrin plate is then prepared as follows:

The hereinafter prepared agarose solution is brought to 53°–56°C and one volume of the solution is homogeneously mixed with one volume of the above-described highly purified fibrinogen solution brought to room temperature. The mixture is then poured into a plate of approximately 3 inches x 1 inch x 1/4 inch deep and allowed to solidify. The plate with the solidified gel is then dipped into the above-described thrombin solution for an appropriate length of time (about 30 seconds) and removed. The plate is immersed in a distilled water bath for an appropriate length of time (about 30 seconds) and then removed. Six wells, each having a diameter of 2 mm. and being equidistantly spaced apart, are then punched into the gel in the clotted plate. The plate is then capped with a protective membrane, packaged and stored at 2°C to 8°C.

The clotted fibrin plate is used with plasma or appropriate plasma preparations for making the following quantitative determinations:

**Test I.** Total plasminogen is determined in a streptokinase-activated euglobulin fraction of plasma.

**Test II.** Available plasmin is determined in a streptokinase-activated plasma.

**Test III.** Active plasmin is determined in plasma.

The procedure employed in carrying out the above three determinations is as follows:

**I) TOTAL PLASMINOGEN TEST**

1. Prepare a streptokinase-activated fraction (SK-E fraction).

   a. Dilute the patient's citrated plasma 1:18 with 5°C distilled water.

   b. Adjust the pH to 5.3 with 1.0 N acetic acid.

   c. Centrifuge in a refrigerated centrifuge for 20 minutes.

2. Add 0.1 ml. of the SK-E fraction to 0.9 ml. of the diluted sample taken in 4. a. above.
(d) Redissolve the precipitate with sufficient agarose gel plate buffer to obtain the original plasma volume.
(e) Add 0.1 ml of streptokinase to 1.0 ml of the euglobulin fraction (product of Step 1d).

(2) Prepare an activated euglobulin control by adding 1.0 ml of a euglobulin control containing a known plasminogen activity to 0.1 ml of streptokinase.

(3) To a total of 6 test tubes, add the following amounts of agarose gel plate buffer:

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Agarose gel plate buffer (ml.)</th>
<th>SK-E fraction</th>
<th>Activated euglobulin (ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.25</td>
<td>SK-E control</td>
<td>control</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>SK-E fraction</td>
<td>control</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>SK-E control</td>
<td>control</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>SK-E fraction</td>
<td>control</td>
</tr>
<tr>
<td>5</td>
<td>0.25</td>
<td>SK-E control</td>
<td>control</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>SK-E fraction</td>
<td>control</td>
</tr>
</tbody>
</table>

(4) Incubate the previously prepared SK-E fraction, the activated euglobulin control, and the 6 tubes of agarose gel plate buffer at 37° C. for 10 minutes.

(5) Add the following amounts of incubated SK-E fraction and activated euglobulin control to the tubes of agarose gel plate buffer and mix well:

(6) Open the clotted fibrin plate; remove and discard the protective membrane from the agar surface.

(7) Fill three wells of the clotted fibrin plate with the activated euglobulin control-agarose gel plate buffer dilutions. Fill the remaining three wells with SK-E fraction-agarose gel plate buffer dilutions. Touch the tip of each capillary to the bottom of the well and allow the solution to flow by gravity so that the well is filled to the level of the agar surface.

(8) Replace the top cover of the plate and incubate at 37° C. preferably in a moist chamber, for approximately 2½ hours, or until clear reaction zones appear for all control dilutions.

(9) Prepare a reference curve as directed below.

(II) AVAILABLE PLASMIN TEST

(1) To prepare activated plasma, add 1.0 ml of the patient's plasma to 0.1 ml of streptokinase.

(2) Proceed as directed in Steps 2-7 of I. Total Plasminogen Test.

(3) Replace the top cover of the plate and incubate at 37° C. preferably in a moist chamber, for at least 4 hours, or until clear reaction zones appear for all activated plasma dilutions.

(4) Prepare a reference curve as directed below.

(III) Active Plasmin Test

(1) To prepare the plasma, add 1.0 ml of the patient's plasma to 0.1 ml of agarose gel plate buffer.

(2) Proceed as directed in Steps 2-7 of I. Total Plasminogen Test.

(3) Replace the top cover of the plate and incubate at 37° C. preferably in a moist chamber, for at least 10 hours, or until clear reaction zones appear for all plasma dilutions.

(4) Prepare a reference curve as directed below.

Preparation of a reference curve

(1) After incubation of the clotted fibrin plate, measure the diameter of each reaction zone by placing the plate, with the cover removed, under the eyepiece of a Hyland Immunoplate® viewer. Align one side of the zone with the zero mark on the grid and measure the diameter of the reaction zone to the nearest 0.1 mm. Alternatively, a dissecting microscope with a stage micrometer or eyepiece reticle may be used.

(2) Using 2-cycle semilogarithmic graph paper, plot the reaction zone diameters of the three controls on the horizontal (arithmetic) scale and the percent activity of the corresponding control on the vertical (logarithmic) scale. Draw a straight line of best fit.

(3) Determine the total plasminogen, available plasmin, or active plasmin of the patient specimen by referring to the line formed by the control. The level of fibrinolytic inhibitor may be calculated by subtracting the available plasmin from the total plasminogen concentration.

EXAMPLE 2

A clotted fibrin plate is prepared as in Example 1, except that a predetermined amount of one unit per ml of plasminogen is added to the highly purified fibrinogen solution. The clotted fibrin plate is then used for making a quantitative determination of plasminogen activator. The procedure employed in carrying out this determination is similar to that employed for Tests II and III in Example 1 except that the control sample of pooled human euglobulin contains additionally the plasminogen activator streptokinase of a known activity. The plasminogen activator in the test specimen is determined by comparison with the control sample as in Example 1.

Various other examples can be devised by the person skilled in the art without departing from the spirit and scope of the invention defined herein. All such further examples are within the scope of the invention as defined in the appended claims.

What is claimed is:

1. A method for the quantitative determination of plasminogen in blood plasma and fractions thereof comprising reacting said blood plasma or fraction thereof with clotted fibrinogen homogeneously suspended in a stable gel medium at a concentration of from about 10 mg. percent to about 2 gm. percent together with a predetermined amount of plasminogen for a predetermined period of time to produce a clear radial diffusion zone of reaction product, measuring the diameter of said radial diffusion zone and comparing with control samples of known plasminogen activator activity, said blood plasma or fraction thereof being allowed to come into contact with said fibrinogen and plasminogen for reaction therewith by diffusion from a well in the surface of said gel on a plate.

2. The method of claim 1 in which the gel medium contains from about 1.0% to about 10% by weight of a gelling agent.

3. The method of claim 2 in which the gelling agent is agarose.

4. The method of claim 3 in which the gel medium contains about 2.5% by weight of agarose.

5. An agarose gel plate for the quantitative determination of plasminogen activator in blood plasma and fractions thereof comprising a plate and an agarose gel medium containing from about 10 mg. percent to about 2 gm. percent clotted fibrinogen together with a predetermined amount of plasminogen homogeneously suspended in said gel medium.