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3,284,301

PLASMIN STERILIZATION

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This invention relates to a method of sterilizing plasmin and, more particularly, to a process for destroying the hepatitis virus in plasmin derived from human placental tissue or human plasma.

It is well established that a proteolytic enzyme is present in normal blood in the form of an inert precursor, plasminogen, which is present in the globulin fraction, for example, Cohn, Fraction III. Plasminogen, when activated by bacterial activators such as staphylokinase, streptokinase, or by tissue activators such as fibrinolysin, or by urine activators such as urokinase or by chloroform in the presence or absence of glycerol, or by autocatalysis, is converted into the proteolytic enzyme plasmin which attacks fibrin and other proteins.

The intrathecal use of plasmin to lyse tuberculosis exudates in vivo has been reported by Fletcher in the Journal of Clinical Investigation, 33, 1242-1251 (1954). Margulis has described the use of plasmin to liquefy blood clots in thirty-eight patients [Archives of Surgery, 65 511-521 (1952)]. Plasmin also finds use in the debridement of wounds, burns, in the treatment of chronic ulcers, and as an anti-inflammatory agent. Contamination of plasmin with the hepatitis virus has constituted a serious health hazard and a bar to its widespread use by the medical profession. Hepatitis is a problem inherent in the use of any medicament derived from the human.

It is an object of the present invention to prepare plasmin which is safe for human use by virtue of its freedom from contamination with the hepatitis virus.

It is still another object of this invention to prepare a sterile plasmin having a high degree of activity.

The existing methods of destroying the hepatitis virus are nitrogen mustard, β -propiolactone, high energy irradiation and heat. Nitrogen mustard and β -propiolactone are of doubtful value and while high energy irradiation in appropriate dosage is effective when applied to products derived from human blood, it may destroy biologically active materials at the intensity necessary for sterilization.

On the other hand, while heat, 60° C. for 10 hours, or heat varying in an inversely proportional manner to time has been of value on known infected biological materials, still it is well known in the art that certain biological materials when heated at these temperatures and durations are unstable resulting in denaturation and loss of biological activity.

In accordance with the present invention, we have discovered a method for heat treating plasmin which results in the destruction of the hepatitis virus with no attendant loss of activity of the plasmin. In addition, the plasmin retains the same degree of solubility both before and after heat treatment. It is also not necessary to add extraneous stabilizing agents to the solution during the sterilization process; a pH adjustment normally suffices.

We take plasminogen isolated from human placental tissue or from human plasma, or from dried plasma fractions, preferably obtained by the method of Kline et al. [Journal of Biological Chemistry, 236, 3232 (1961)], or by the methods of Hagen et al., United States Patents Nos. 2,923,665 and 3,066,079. It should be pointed out, however, that the degree of purity of the plasminogen or the manner in which purification is achieved is immaterial so

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far as achieving the objects of the present invention is concerned.

The plasminogen is converted to plasmin by any of the well-known activators such as urokinase or streptokinase. Stabilizers such as glycerol [J. Biol. Chem. 233, 81 (1958), and J. Biol. Chem. 233, 86 (1958)], may be employed during the activation as described by Alkjaersig et al.

After conversion of plasminogen, obtained by any of the above described procedures, to plasmin, the resulting plasmin can be readily dissolved in concentrations of less than 10% in an aqueous solution between pH 0.3-4.5 and 7-11.0. Between pH 5.0 and 7.0 the plasmin is less soluble. For the purpose of our invention it is desirable to dissolve the plasmin between pH 0.3-8.0 and preferably at pH 3-4.0, the appropriate adjustment being made with an acid such as hydrochloric acid. Below about pH 3.5 and above about pH 4.5 the plasmin solutions are less stable to the sterilization process and significant losses of plasmin activity are incurred.

Thereafter the solution is heated at 60° C. for 10 hours and if it is desired, the clear solution may be sterile filtered when cool. In order to render the plasmin solution neutral after heating, if necessary, lysine monohydrochloride or any other suitable solubilizing agent as described in United States Patent No. 3,066,079 is added, up to 0.1 M final concentration, and then a base such as tris(hydroxymethyl)aminomethane, sodium hydroxide or sodium carbonate is added to raise the pH to between 6.0-9.0. Sterile filtration may thereafter follow in the usual manner.

The invention will be described in greater detail in conjunction with the following specific examples.

EXAMPLE I

Plasminogen is prepared from human serum, dried Fraction III by the procedure of Kline and Fishman [J.B.C. 236, 3232 (1961)], and activated to plasmin by streptokinase in the presence of glycerol. The specific activity of the plasmin is 14.7 caseinolytic units per mg. of protein and a solution of the preparation contains 2.6 mg./mm. 1.25 parts of the plasmin solution are mixed with 23.5 parts of 1×10^{-4} M HCl and the pH is adjusted to 4.0 with 1 N HCl. The solution is divided into two parts. Part A is heated at 60° C. for 10 hours in a loosely covered tube, then cooled at 5° C. Part B, to be used as a control, is stored at 5° C. until the heat treatment on Part A is concluded. Both parts are then individually adjusted to a concentration of 0.1 molar in lysine monohydrochloride, then adjusted to a concentration of 0.06 molar in tris(hydroxymethyl)aminomethane, which is added and mixed rapidly, to bring the pH to 8.0, for purposes of assay. The solutions are analyzed by the direct caseinolytic assay as shown in Example IV below. Part B, the unheated control is found to have all its starting activity. Part A, the heat-treated solution, is found, surprisingly, to have retained its full initial activity.

EXAMPLE II

Plasminogen is prepared from placental waste Fraction III₂ by the procedure of Kline and Fishman and activated to plasmin by streptokinase. The specific activity of the plasmin is 5.2 units/mg. protein, and a solution of the preparation contains 3.6 mg./ml. One part of the plasmin solution is added to 5 parts of 1×10^{-4} M HCl and the pH is adjusted to 4.0 with 1 N HCl. The solution is divided into two parts, A and B. Part A is heated at 60° C. for 10 hours in a loosely covered tube, then cooled to 5° C. Part B, to be used as a control, is stored at 5° C. until the heat treatment of Part A is concluded. At this point, both solutions are, respectively made 0.1 molar in lysine monohydrochloride and 0.06 molar in tris(hy-

droxymethyl)aminomethane which is added and mixed rapidly, to bring the pH to 8.0 for purposes of assay. The solutions are analyzed by the direct caseinolytic assay as shown in Example IV below. Part B, the control plasmin solution which is unheated is found to have all its starting activity. Part A, the heat-treated plasmin solution is found to have 80 percent of the activity of the control.

EXAMPLE III

Example II is repeated except that the starting plasmin solution (5.64 mg. protein/ml.) is not diluted. It is adjusted to pH 3.7 and heated at 60° C. for 10 hours. The recovery of plasmin in the heat-treated sample, is found to be 85 percent of the activity of the control.

EXAMPLE IV

A. Reagents

(1) Azocasein 40 mg./ml. or 4.0 gm./100 ml.; Sorensen buffer at pH 7.5. Add dry to a blender to break up the azocasein. Add the Sorensen buffer and blend for 15 seconds at high speed. Let the foam settle for 15 minutes and centrifuge in Sevall centrifuge at 30,000 G for 20 minutes.

(2) Sorensen buffer:	Gms.
Na ₂ HPO ₄ ·12H ₂ O -----	9.52
KH ₂ PO ₄ -----	0.907
NaCl -----	0.45

(Make up to 1 liter and adjust pH to 7.5)

(3) 1.7 M perchloric acid: 200 ml. of 60 percent HClO₄ is made up to 1.0 liter with distilled water.

(4) Samples: pH must be between 7 and 8.5 and the material should be in solution. If the samples are on the acid side of the isoelectric point add solid lysine to a final concentration of .05 M to 0.1 M or if a dilution is being made use .05 M or .1 M lysine as a diluent. Adjust the pH up with solid tris to a final concentration of .04 to .08 M, depending on the initial pH of the solution. The solution should be mixed immediately on adding the tris.

B. Method

Arrange 12 ml. centrifuge tubes in a rack.

Example for 6 assays.—Use 6 rows of four tubes each. First tube is zero time, second tube is 10 minutes time, third is 15 minutes, and the fourth is the 20 minutes time tube. Add 3 ml. of 1.7 M HClO₄ to all tubes. Set water bath to 30° C. with stirring. Add 1.0 ml. of azocasein solution to the 6 zero time tubes (first tube in each row) and stir on a mixer. These are for the zero time blanks.

(1) Begin the assay: Put 6 test tubes of 25 ml. size (these are the reaction tubes) one in back of each 20 minute time centrifuge tube. To the first of these reaction tubes add 2.0 ml. of the sample to be analyzed. The other 5 samples are added to their respective reaction tubes at 10 second intervals so that 50 seconds has elapsed between the addition of the first sample to the first reaction tube and the addition of the sixth sample to the sixth reaction tube.

(2) 2.3 ml. Sorensen buffer is immediately added to the first reaction tube and at 10 second intervals the same amount of buffer is added to the other reaction tubes.

(3) 1.0 ml. is immediately withdrawn from reaction tube one and added to the zero time tube for that sample by blowing out the pipette to achieve mixing. 10 seconds later withdraw 1.0 ml. from reaction mixtures two

and add it to the zero time tube for sample two. Do the same for the other reaction mixtures. Stir on mixer. These are the zero time blanks.

(4) 3.3 ml. of the azocasein solution is added to the first reaction tube and a clock started. At 10 second intervals add 3.3 ml. of azocasein to the other reaction tubes and mix by hand.

(5) After 10 minutes withdraw 2.0 ml. from the first reaction tube and add it to the 10 minute time centrifuge tube for the sample (the centrifuge tube directly behind the zero time tube). Blow in the sample and stir on mixer. At 10 second intervals do the same for the other reaction tubes.

(6) After 15 minutes repeat step five by putting 2.0 ml. from the reaction tubes into the appropriate 15 minute time centrifuge tube.

(7) After 20 minutes repeat step five by putting 2.0 ml. from the reaction tube into the appropriate 20 minute time centrifuge tube.

(8) Let sit one-half hour at room temperature after the additions to the 20 minute tubes.

(9) Filter through whatman No. 12 folded filter paper in a glass cone funnel.

(10) Read optical density in the Beckman DU spectrophotometer with hydrogen lamp at 350 mμ.

C. Calculations

(1) Plot optical density at 350 mμ vs. time. Draw the best straight line between these points.

(2) The optical density at the 20 minute time is read from the curve. The blank is subtracted from the O.D. at 20 minutes. This difference is divided by 20 to give the rate of change in the O.D. per minute. This result is divided by 2 to give the ΔO.D./min. for 1 ml. of the sample solution (2 ml. of the sample are used in the assay). This value is then multiplied by 1000 as a convenience factor in order to express the unitage in numbers greater than 1.

(3) The result is units per ml. of the sample analyzed. If the sample is a dilution, then the u./ml. are multiplied by the dilution factor to give the u./ml. of the original solution.

(4) The unit is defined then as:

$$\frac{(\text{O.D. 20 minutes} - \text{O.D. 0 minute})}{20 \text{ minutes}} \times \frac{1000}{2}$$

We claim:

1. A method of treating plasmin which comprises heat-treating at a temperature of about 60° C. for about 10 hours an aqueous solution of not more than 10 percent by weight of plasmin having a pH between about 0.3–4.5.

2. A method as in claim 1 in which the plasmin is derived from human plasma plasminogen.

3. A method as in claim 1 in which the plasmin is derived from human placental plasminogen.

4. A method as in claim 1 in which the aqueous solution of plasmin has a pH of about 4.

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