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PRODUCTION OF STABLE HIGH-POTENCY HUMAN AHF USING POLYETHYLENE GLYCOL AND GLYCINE TO FRACTIONATE A CRYOPRECIPIRATE OF AHF CONCENTRATE

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8 Claims

ABSTRACT OF THE DISCLOSURE

The production of a stable antihemophilic factor of high potency by the fractionation of a cryoprecipitate concentrate of antihemophilic factor.

This application is a continuation of application Ser. No. 679,240, filed Oct. 30, 1967, now abandoned, which was a continuation-in-part of application Ser. No. 634,839, filed May 1, 1967, and now abandoned.

This invention relates to a method of making a concentrate of antihemophilic factor (AHF, Factor VIII).

The process of blood coagulation is an important activity which normal whole blood is capable of carrying out under timely circumstances to prevent excess loss of blood through open wounds or by internal bleeding. It is known that normal whole blood contains a factor which is absent or seriously deficient in hemophiliacs. This factor is associated with the globulin fraction of blood and has come to be known as antihemophilic factor (AHF, Factor VIII).

Scientists have known about AHF and its role in blood coagulation for some time, and treatment of hemophilia heretofore has generally consisted of replacement therapy whereby the patient is transfused with many pints of fresh whole blood or specially prepared plasma.

It is known, however, that under ordinary storage conditions whole blood and liquid plasma lose their AHF activity in a day or so. While it is possible to freeze and store fresh plasma, AHF activity in frozen plasma has a relatively short life of several months or so.

It is also possible to decrease the loss of AHF activity during storage by drying freshly frozen plasma, but it has not heretofore been possible to know with certainty the strength of AHF in this dried material since the amount of AHF in normal individuals who donate the plasma varies widely, from about 50% to about 200% of average.

Conventional replacement therapy also suffers from other serious disadvantages since the hemophiliac often develops allergic or refractory states when repeatedly treated with plasma. Moreover, the large amounts of plasma needed by the hemophiliac tend to cause hypervolemia or overloading of the circulatory system. Such overloading puts a strain on the heart and threatens the patient with heart failure.

Scientists have also proposed various methods for the isolation of AHF or the preparation of plasma fractions rich in AHF from human or animal blood. In practice, these methods heretofore have provided to be unreliable since the AHF activity of the fractions tends to be lost during the isolation. AHF is a labile trace protein which is difficult to separate completely from other plasma proteins, particularly fibrinogen, and the yield of AHF from the plasma by these previous methods has not been high. The AHF potencies of previously prepared blood

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fractions for hemophiliac therapy are known to be low and the cost of treatment is high.

Other drawbacks in prior attempts to isolate AHF are that AHF is readily susceptible to denaturation by heat, freezing and thawing, and continued storage.

Among the various methods that have been heretofore proposed for the isolation of AHF are chromatography, batch absorption and elution, and selective precipitation. Various precipitating agents that have been used are ethanol, ethyl ether, ammonium sulfate, phosphate-sodium citrate, amino acids, and cryoprecipitation procedures. Recently, the clinical use of a glycine-precipitated AHF fraction of whole plasma was disclosed by Webster et al., Amer. J. Med. Sciences, vol. 250, No. 6, pp. 643-650 (1965), and the clinical trials of AHF replacement therapy with a cryoprecipitated fraction of whole plasma in a closed-bag system was reported by Pool et al., New England J. Med., vol. 273, No. 27, pp. 1443-1447 (1965). None of the above methods, however, has proved to be a completely practical method for isolating AHF.

In accordance with the present invention, as distinguished from the prior art, a method for the preparation of a stable AHF of high potency is provided which comprises the fractionation of a cryoprecipitate concentrate of human or animal AHF.

As used herein the term "cryoprecipitate concentrate of AHF" refers to the precipitate obtained from the freezing of human or animal blood plasma at $\leq 4^{\circ}$ C. and separated from the supernatant. It is preferred that this cryoprecipitate concentrate of AHF is obtained by rapid freezing of fresh plasma, but stored plasma can also be used as source material in the practice of the present invention. It is also preferred to carry out the freezing to a temperature of from about -20° C. to about -40° C. followed by slow thawing to about 4° C.

In addition to the stability and high potency of AHF activity obtained by the method of the present invention, use of the cryoprecipitate concentrate of AHF rather than whole plasma as the starting material has the advantage of allowing retention of other plasma components such as albumin, gamma globulin and the like which are usually destroyed when whole plasma is purified by previously known methods of preparing AHF fractions for use in treatment of hemophilia.

In accordance with the present invention the cryoprecipitate concentrate of AHF preferably is fractionated to yield a stable AHF of high potency by means comprising one or more of the following steps:

- (a) precipitation with glycine,
- (b) precipitation with polyethylene glycol.

In the glycine precipitation step, the cryoprecipitate concentrate of AHF is first redissolved and then the redissolved fraction is precipitated with an aqueous solution of glycine having a molarity of from about 1.3 to about 1.8, followed by recovery and redissolution of the precipitate.

Recovery of the cryoprecipitate concentrate and the glycine-precipitated fraction for use in this invention can be accomplished, for example, by centrifugation or filtration of the respective precipitates or by similar such procedures.

Redissolution of the recovered precipitates can be achieved by warming and agitating in citrated saline solution. In the case of the redissolution of the cryoprecipitate concentrate, it is preferred to use a glycine citrated saline solution and to increase the volume of the cryoprecipitate concentrate to about one-tenth the volume of original plasma the cryoprecipitate concentrate represents. The glycine-precipitated fraction is preferably redissolved with citrated saline solution to increase the volume of the frac-

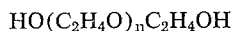
tion to about one-twentieth the volume of plasma the glycine-precipitated fraction represents.

It is also preferred to purify each of the respective redissolved cryoprecipitate concentrate and glycine-precipitated fraction by clarifying with additional centrifugation and/or filtration to remove any insoluble matter.

The above fractionation of a cryoprecipitate concentrate of AHF by precipitation with glycine has been found to provide an AHF concentrate of high potency which can be frozen and rendered stable, such as by lyophilization, and retained under ordinary refrigeration conditions for periods of a year or longer. The potency of each batch of material prepared by the above fractionation method can be precisely determined so that the administering physician can know for the first time exactly how much AHF his patient receives.

Since the redissolved AHF concentrate prepared by the above fractionation method has more than five times the AHF activity of an equal volume of plasma, the hemophiliac can be given a quantity of AHF which the heart could not otherwise tolerate. Even more importantly, the AHF activity in the above-prepared concentrate is contained in less than one-fifteenth the amount of protein present in plasma providing an equal amount of AHF activity. This lower protein content minimizes the likelihood of allergic reactions by the hemophiliac recipient and reduces the possibility of overloading the circulatory system.

Another example of means which can be used to fractionate the cryoprecipitate concentrate of AHF according to the present invention is precipitation with polyethylene glycol. Polyethylene glycols are high molecular weight polymers which are generally produced by reacting ethylene oxide with ethylene glycol or water and have the following structure:



in which n represents the average number of oxyethylene groups. According to the present invention the polyethylene glycols should be nontoxic and can range in molecular weight from about 200 to about 20,000. They preferably have molecular weights from about 400 to about 6,000. PEG 4000, which is a polyethylene glycol product having an average molecular weight of about 4,000, is the preferred product of this group.

In the polyethylene glycol precipitation step it is preferred to use from about 3% to about 4% polyethylene glycol by weight of the redissolved AHF concentrate with retention of the supernate, followed by use of about 10% polyethylene glycol by weight of the supernate with retention of the precipitate. The latter precipitate is then recovered and redissolved in citrated saline.

The polyethylene glycol precipitation step can also be employed in combination with the glycine precipitation step. If the polyethylene glycol precipitation step precedes the glycine precipitation step, the molarity of the glycine used for the precipitation should be about 1.8.

In the aforesaid combination of precipitation steps, it is preferred that the glycine precipitation step follow the polyethylene glycol precipitation step. It has been found that the glycine helps to remove any residual polyethylene glycol remaining in the concentrate of AHF and thereby reduces any potential danger from the presence of polyethylene glycol. Moreover, the concentrate of AHF prepared in this manner has been found to be substantially more soluble than the like product in which the glycine precipitation step preceded the polyethylene glycol precipitation step.

The AHF concentrate prepared by the successive precipitation steps with glycine and polyethylene glycol as hereinbefore described can be frozen and rendered stable, such as by lyophilization, and retained under ordinary refrigeration conditions for periods of a year or longer. The reconstituted AHF concentrate has more than thirty times the AHF activity of an equal volume of plasma; and

the AHF activity of this concentrate is contained in less than one hundredth the amount of protein present in plasma providing an equal amount of AHF activity.

The cryoprecipitate concentrate of AHF which has been fractionated by glycine precipitation and/or polyethylene glycol precipitation can be further treated by purification with ECTEOLA cellulose resin. This purification can be carried out either before or after the glycine and/or polyethylene glycol precipitation and may be done by column or batch techniques. The concentrate purified by this method has the additional advantage that it can also be administered intramuscularly as well as by the intravenous administration methods generally used in the case of the AHF concentrate which has not been treated with the ECTEOLA cellulose resin. The AHF concentrate purified with ECTEOLA cellulose resin has been found to be free of fibrinogen by the addition of thrombin and by immunoelectrophoresis.

As used herein, the term "ECTEOLA cellulose resin" refers to a modified cellulose which contains active basic substituents introduced into the cellulose molecule by reaction with epichlorohydrin and triethanolamine. Methods of preparation of ECTEOLA cellulose resins are described in general by Sober and Peterson, *J. Am. Chem. Soc.*, vol. 76, pp. 1711-12 (1956); *id.*, vol. 78, pp. 751-55 (1956); vol. 78, pp. 756-63 (1956); and Peterson and Sober, *Biochem. Preparations*, vol. 8, pp. 43-4 (1961).

ECTEOLA cellulose resins are available commercially. However, it has been found desirable to initially treat these resins by recycling them with caustic soda before use in the herein-defined purification procedure.

In the purification procedure with ECTEOLA cellulose resin, the resin preferably is first equilibrated with a chloride buffer solution having a concentration of about 0.8% NaCl and then poured into a chromatographic glass column. The AHF concentrate which is desired to be purified is then applied to the column and finally eluted with a chloride buffer solution having a molarity of about 0.5.

Other methods of purifying the AHF concentrate of the present invention will be apparent to those skilled in the art.

The following examples further illustrate the present invention although the invention is not limited to these specific examples which are provided for purposes of illustration and not limitation. All parts and percentages herein are on a weight basis unless otherwise specified.

EXAMPLE 1

A stable human AHF concentrate of high potency is prepared by the successive fractionation of human blood plasma, first by cryoprecipitation and then by glycine precipitation in the following manner:

Reagents

Citrated saline.—One part 0.1 molar sodium citrate to four parts by weight 0.9 percent saline.

Glycine citrated saline.—Sufficient glycine is added to the above citrated saline to make a 0.1 molar solution respective of glycine.

Buffered wash water.—To distilled water add $\frac{1}{100}$ volume of buffered citrate which is made by adjusting 0.5 molar sodium citrate with 0.5 molar citric acid to pH 6.88.

Acetic acid.—Prepared both 1.0 normal and 0.1 normal aqueous solutions.

Glycine.—Prepare 1.3 molar aqueous solution.

Procedure

Human blood plasma is received frozen ($\leq 4^\circ \text{C.}$) from a donor center. The plasma is pooled into Pfaunder kettles and, while held at a temperature of less than 4°C. , it is centrifuged by continuous flow or bucket centrifugation. The resulting cryoprecipitate is collected and retained for further fractionation hereinafter in accordance with the present invention.

To the cryoprecipitate, glycine citrated saline is added, the amount being one-tenth the volume of plasma the cryoprecipitate represents. Dissolution is brought about by mixing the cryoprecipitate and glycine citrated saline in a warm environment (room temperature, but not in excess of 30° C.).

When the cryoprecipitate has dissolved, it can be clarified, if desired (depending upon the amount of red cells and denatured protein present), by further centrifugation and/or filtration.

The dissolved cryoprecipitate is then adjusted to pH 6.88 with 0.1 normal acetic acid. By suitable means (for example, refrigeration or use of isopropanol Dry Ice bath) the solution is cooled to a temperature of from 6° C. to 10° C. To the cooled solution, sufficient glycine is added to make the solution 1.2 molal with respect to glycine. The mixture is gently agitated for 45 to 60 minutes at a temperature of from 2° C. to 10° C., and then centrifuged by continuous flow or bucket centrifugation. The resulting glycine precipitate is collected and gently washed with buffered water at a temperature of 0° to 4° C.

When the glycine precipitate has dissolved, it is preferable to clarify the solution by centrifugation and/or filtration using a 293 mm. Millipore filter (membranes used: 1.2 microns, 0.45 micron, and 0.3 micron).

The liquid blood plasma product prepared in the above manner by the successive fractionation, first by cryoprecipitation and then by glycine precipitation, has an AHF concentration of high potency. This liquid product is then frozen by shell freezing (−60° C.) and storing in a flash freezer (−20° C. to −30° C.) for at least three hours. The frozen product can then be retained under ordinary refrigeration conditions (≤4° C., preferably at −20° C. to −30° C.) without loss of its AHF activity for periods of time of one year and longer. This product when reconstituted for administration contains five times the AHF activity of an equal volume of normal blood plasma and is contained in only one-fifteenth the amount of protein present in plasma providing an equal amount of AHF activity. The reconstituted product can be administered intravenously to hemophiliacs as required by conventional transfusion means.

EXAMPLE 2

Example 1 is repeated up to and including the step of adding the citrated saline to the glycine precipitate. The redissolved precipitate is adjusted to pH 6.5 with 1.0 normal acetic acid. Polyethylene glycol 4000 is added to the solution to make the PEG concentration 3.5 percent. The mixture is gently agitated at room temperature for ten minutes, and then centrifuged for fifteen minutes at 5000 r.p.m. The supernate is decanted and adjusted to pH 6.88 with 1.0 normal sodium hydroxide. Additional polyethylene glycol 4000 is added to the solution to make the final PEG concentration 10 percent. The mixture is gently agitated at room temperature for thirty minutes, and centrifuged at 5000 r.p.m. for one-half hour. The supernate is decanted and the precipitate is washed in cold water (2° C.). Spin washing is then carried out for five minutes at 5000 r.p.m. at a temperature of −4° C. The supernate is decanted and the precipitate is dissolved in citrated saline. The clarified solution is filtered using a 293 mm. Millipore filter as described in Example 1. This final liquid product can be frozen by shell freezing, followed by storage in a flash freezer for at least three hours, and then retained under ordinary refrigeration conditions for subsequent use in hemophiliac therapy in the manner of the final product of Example 1.

EXAMPLE 3

Example 2 is repeated including the additional step of purification of the AHF fraction with ECTEOLA cellulose resin in the following manner.

Reagents

ECTEOLA cellulose resin—

- (1) Mix 60 gm. NaOH with 150 ml. H₂O.
 - (2) Allow mixture to cool.
 - (3) Place 60 gm. cellulose (Whatman Cellulose Powder CF 11) in a beaker and mix thoroughly with above NaOH solution.
 - (4) Allow mixture to stand overnight (12 hours).
 - (5) Next day prepare a solution of 35 ml. triethanolamine and 60 ml. of epichlorohydrin. Mix well under a hood.
 - (6) Add this solution quickly to the above cellulose and mix well. Place the reaction vessel out of draft. (The reaction is exothermic and will heat to about 100° C. The mixture will turn brown in one to two hours.)
 - (7) Cool the mixture at room temperature under hood.
 - (8) Add 350 ml. 2 M NaCl in small portions.
 - (9) Filter this mixture through a coarse sintered glass filter.
 - (10) Wash precipitate twice with 500 ml. of 1 N NaOH. (This removes deep discoloration.)
 - (11) Suspend precipitate in 350 ml. 1 N HCl in filter funnel. Apply vacuum.
 - (12) Repeat step 11 with 250 ml. 1 N NaOH.
 - (13) Repeat step 11 with 250 ml. 1 N HCl.
 - (14) Repeat step 11 with 250 ml. 1 N NaOH.
 - (15) Transfer precipitate to a 3 liter beaker.
 - (16) Add 250 ml. 1 N NaOH. Mix.
 - (17) Add distilled water to mixture to fill beaker; mix. Cover and allow to stand overnight.
 - (18) Decant supernatant.
 - (19) Add water to precipitate to fill beaker and mix.
 - (20) Wash mixture on the filter with water. (Four liters or more, until a negative test for alkali with 1% alcoholic phenolphthalein is obtained.)
 - (21) Make a final wash with two 250 ml. portions absolute ethanol.
 - (22) Place on filter paper. Mash and spread and place it to dry overnight.
- Chloride buffer—
- 0.8% NaCl (8 gm./l.)
0.02 M Imidazole (1.36 gm./l.)
Adjust pH to 6.9 with 1 N HCl
- Elutting buffer—
- 0.5 M NaCl
0.02 M Imidazole (1.36 gm./l.)
Adjust pH to 6.9 with 1 N HCl
- A commercially available ECTEOLA cellulose resin which is first recycled with NaOH, for example, as in the following manner, can be used in place of the above-prepared ECTEOLA cellulose resin.
- Recycled commercial ECTEOLA cellulose resin—
- (1) Mix 60 gm. commercial ECTEOLA cellulose resin with 350 ml. 2 M NaCl.
 - (2) Filter this mixture through a coarse sintered glass filter.
 - (3) Wash precipitate two times with 500 ml. 1 N NaOH.
 - (4) Wash one time with 350 ml. 1 N HCl.
 - (5) Wash one time with 250 ml. 1 N NaOH.
 - (6) Wash one time with 250 ml. 1 N HCl.
 - (7) Wash one time with 250 ml. 1 N NaOH.
 - (8) Transfer precipitate to a 3 liter beaker and add 250 ml. 1 N NaOH. Mix.
 - (9) Add distilled water to mixture to fill beaker. Mix. Cover and let stand overnight.
 - (10) Decant liquid; add 3 liters water to precipitate; mix.
 - (11) Filter.
 - (12) Wash precipitate with water until phenolphthalein test is negative.
 - (13) Wash precipitate two times with 500 ml. absolute EtOH.
 - (14) Air-dry precipitate on filter paper.

Procedure

When the starting material for the purification with ECTEOLA cellulose resin is the cryoprecipitate concentrate of AHF or the polyethylene glycol-precipitated fraction of AHF, the AHF fraction is first dissolved in chloride buffer. When the starting material is the glycine-precipitated fraction of AHF, the AHF fraction is first dialyzed against the chloride buffer for one hour to remove glycine and reduce its ionic strength. Purification of the buffered AHF fraction by column technique with the above-prepared ECTEOLA cellulose resin proceeds as follows:

The ECTEOLA cellulose resin is equilibrated overnight (12 hours) in a 5° C. box by mixture with chloride buffer in proportions of 15 gm. resin to 600 ml. buffer. The resulting resin slurry is poured on a column of from one to 1½ inches in diameter x 18 inches high. After the buffer comes down to the level of the resin, the AHF fraction is adjusted to 2½ ml. per minute and the amount of AHF fraction applied to a single column is from 1000 to 2500 units of AHF. (One unit of AHF is equal to the AHF activity in one cc. of normal human blood plasma.) After the AHF has been applied to the column, from 200 to 500 ml. chloride buffer is washed through the resin. When the chloride buffer comes down to the level of the resin, the eluting buffer is applied to the column. The eluate is collected in ten ml. portions and analyzed for protein fibrinogen and AHF activity.

The eluate portions having the most active AHF activity are retained and stabilized by the addition of 1% albumin. The stabilized solution is then filtered using a 293 mm. Millipore filter as in Example 1. A silver filter of the same size can also be used in place of the Millipore filter. This final liquid product can then be frozen by shell freezing, followed by storage in a flash freezer for at least three hours, and then retained under ordinary refrigeration conditions in the manner of the final product of Example 1.

EXAMPLE 4

Example 1 is repeated up to and including the step of dissolving the cryoprecipitate in glycine citrated saline. The dissolved cryoprecipitate is adjusted to pH 6.5 with 0.1 normal acetic acid. Polyethylene glycol 4000 is added to the solution to make the PEG concentration 3.5 percent. The mixture is gently agitated at room temperature for ten minutes, and then centrifuged for fifteen minutes at 5000 r.p.m. The supernate is decanted and adjusted to pH 6.88 with 0.1 normal sodium hydroxide. Additional polyethylene glycol 4000 is added to the solution to make the final PEG concentration 10 percent. The mixture is gently agitated at room temperature for thirty minutes, and centrifuged at 5000 r.p.m. for one-half hour. The supernate is decanted and the precipitate is washed in cold water (2° C.). Spin washing is then carried out for five minutes at 5000 r.p.m. at a temperature of -4° C. The supernate is decanted and the precipitate is redissolved in citrated saline.

The redissolved precipitate is adjusted to pH 6.88 with 0.1 normal acetic acid and then reprecipitated with glycine according to the procedure of Example 1 except that the molarity of the glycine reagent is 1.8. The glycine precipitate is washed and clarified, and then frozen as in Example 1.

The AHF concentrate prepared by the procedure of this example contains less than 0.05% (generally as little as 0.01%) residual polyethylene glycol whereas the AHF concentrate prepared by the procedure of Example 2, above, contains on the order of about 0.5% polyethylene glycol. The final product made by the procedure in this example also is substantially more soluble than the like product prepared in Example 2, above. This concentrate has more than thirty times the AHF activity of an equal volume of plasma; and the AHF activity of this concentrate is contained in less than one hundredth the amount of protein present in plasma providing an equal amount of AHF activity.

Various modifications and adaptations of the present invention can be devised, after reading the foregoing specification and the claims appended hereto, by the person skilled in the art without departing from the spirit and scope of the invention. All such variations and modifications are included within the scope of the invention as defined in the following claims.

What is claimed is:

1. In the method of preparing a concentrate of AHF, the improvement comprising the fractionation of a cryoprecipitate concentrate of AHF with both polyethylene glycol and glycine, said cryoprecipitate concentrate of AHF being obtained as the precipitate from the freezing of blood plasma at $\leq 4^{\circ}$ C. followed by thawing and separated from the supernatant, said fractionation with polyethylene glycol comprising two successive precipitations with polyethylene glycol having a molecular weight of from about 200 to about 20,000, first to a concentration of from about 3% to about 4% by weight of polyethylene glycol followed by discardal of the resulting precipitate and recovery of the supernate, and then to a concentration of about 10% by weight of polyethylene glycol followed by recovery of the resulting precipitate and discardal of the supernate, said fractionation with glycine comprising precipitation with aqueous glycine having a molarity of from about 1.3 to about 1.8 followed by recovery of the resulting precipitate and discardal of the supernate.

2. The method of Claim 1 in which the fractionation with polyethylene glycol precedes the fractionation with glycine.

3. The method of claim 2 in which the glycine has a molarity of about 1.8.

4. The method of claim 1 in which the polyethylene glycol has an average molecular weight of from about 400 to about 6000.

5. The method of claim 1 in which the polyethylene glycol has an average molecular weight of about 4000.

6. The method of claim 1 including the additional step of purification with triethanolaminoethylated cellulose resin.

7. The method of claim 1 in which the fractionation with polyethylene glycol precedes the fractionation with glycine, the polyethylene glycol has an average molecular weight of about 4000 and the glycine has a molarity of about 1.8.

8. The method of claim 7 including the additional step of purification with triethanolaminoethylated cellulose resin.

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