

PATS 1 - DATA SHEET

UK Appln No Filed	Patent No	File No D/PD Pats/P/0186	Security Grading UNCLASSIFIED
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Inventor I WinkelmanGROUP Pats 1A(3)Establishment or Branch (Plasma Fractionation Lab
Churchill Hospital, Oxford)AGENT R F WalkerAssigned to Central Blood Laboratories AuthorityShort Title Separation of Factor VIIIResume

This invention relates to a method of purifying the protein Antihæmophilic Factor VIII from the blood plasma fraction cryoprecipitate by adding at least 0.15 mg herapin per ml of the fraction to precipitate out the unwanted proteins fibrinogen and fibronectin. After separating out the precipitant the supernatant may be pasteurised to inactivate any viruses present, and then may be further concentrated by a series of known steps to produce freeze-dried Factor VIII concentrate.

The invention compares very favourably with other known methods of fibrinogen/fibronectin precipitation and gives rises to consistently high yield of Factor VIII.

Signed R LUETCHFORD

Date 5 March 1985

The patent application number should be quoted as a reference in any report or paper containing a description of this invention and should be mentioned to interested firms, particularly collaborators and contracts.

Patents 1 should be advised of direct communications abroad and to overseas visitors.

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Agents ref: D/PD Pats/P/0186

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PURIFICATION OF BLOOD COAGULATION FACTOR VIII BY PRECIPITATION

This invention relates to the purification of factor VIII (Antihæmophilic Factor, AHF) from blood plasma concentrates, especially cryoprecipitate.

Blood clotting factor VIII is a protein component of blood which has for many years been used to treat individuals suffering from classical hæmophilia (hæmophilia A), a congenital disease caused by a deficiency or absence of factor VIII in the blood. Until the 1960s treatment of hæmophiliacs consisted of transfusing the patient with whole blood or blood plasma. However, in the last 10 to 20 years, factor VIII - enriched plasma protein concentrates have increasingly replaced these whole plasma transfusions and have increased the effectiveness of anti-hæmophilia treatment.

The most commercially important of the plasma concentrates currently used are the blood plasma fraction commonly known as cryoprecipitate, and more purified concentrates prepared from it. Conventionally, cryoprecipitate is defined as a precipitate, rich in factor VIII, fibrinogen (blood coagulation Factor I) and fibronectin (cold-insoluble globulin, CIG), which is prepared from frozen freshly-prepared human plasma by a low temperature plasma fractionation technique. Typically, deep-frozen plasma is softened to -5°C to -15°C , and then warmed slowly under efficient manual or mechanical stirring to a temperature of not more than about 3°C . Under these conditions the frozen plasma partially thaws to yield a liquid phase and a solid phase, and it is this solid phase which is recovered by centrifugation as commercially valuable cryoprecipitate. Cryoprecipitate prepared in this way typically contains concentrated within it from 40 to 60% of the total amount of factor VIII contained in the whole blood from which the plasma is derived.

There have been numerous studies to improve the yield of factor VIII from cryoprecipitate and other blood plasma fractions, and to stabilise and further purify it. For example, a minimum purification step is the treatment of cryoprecipitate with $\text{Al}(\text{OH})_3$ gels which have been found to eliminate some of the other blood clotting factors, stabilise the activity of factor VIII, and facilitate the subsequent sterile filtration of factor VIII.

The major shortcoming of present technology is that for the most part multi-stage processing of plasma results in rather costly losses of factor VIII activity. In addition, it has long been accepted that commercial preparations of Factor VIII vary greatly in terms of anti-haemophilic activity, total protein content, and contamination by other proteins particularly fibrinogen and fibronectin. Thus, all presently known methods for the purification of factor VIII preparations involve at least partial separation of fibrinogen from factor VIII in order to increase the ratio of factor VIII to total protein content in the preparations.

US Patent No 4406886 (Bier et al) describes one such method of purifying factor VIII in which fibrinogen is precipitated from cryoprecipitate preparations by the addition of zinc ions. However cryoprecipitate and other blood plasma fractions usually contain citrate anticoagulants which are added to fresh blood soon after donation and are carried over into the concentrate. Bier et al acknowledge that the concentration of zinc ions required to cause a given precipitation is dependent on citrate ion concentration, and they further acknowledge that the optimum range of zinc ion concentration for a given separation is rather narrow. In practice, because it is often necessary to obtain cryoprecipitate from plasma in different anticoagulants, and since cryoprecipitate as recovered from the centrifuge contains varying amounts of supernatant fluid, citrate ion concentration within the plasma fraction is variable and is usually unknown at the time of zinc addition. This has resulted in considerable difficulty in obtaining consistent results in terms of both separation of fibrinogen and yields of factor VIII.

A further problem associated with the method of Bier et al is that the conditions used to obtain optimal yield of Factor VIII leave in the solution concentrations of contaminating fibrinogen and fibronectin which limit the success of further processes, such as heating to inactivate viruses.

The present invention seeks to overcome one or both of these problems associated with the process of Bier et al by providing a method for precipitating fibrinogen and fibronectin from blood plasma fractions containing fibrinogen, fibronectin and factor VIII which comprises adding a sulphated polysaccharide (hereinafter

referred to as "SPS") to the blood plasma fraction in a concentration of at least 0.15 mg of the polysaccharide per ml of the fraction.

5 Examples of SPS's suitable for use in the method of the present invention are dextran sulphate and chondroitin sulphate. Preferably, however, the polysaccharide used is heparin.

Heparin is a complex organic acid containing glucosamine, glucuronic acid and sulphuric acid. It is known to delay the coagulation of blood, and is used, usually by intravenous or subcutaneous injection, in medicine and surgery. It is normally
10 obtained from the lungs or intestinal mucosa of mammals, and in the present invention it is preferably used in the form of a water-soluble alkali metal salt, preferably its sodium salt.

The blood plasma fraction is preferably a cryoprecipitate solution comprising cryoprecipitate diluted with between 0.5 and 6 parts, most preferably 1 to 3 parts, of an aqueous solution. Cryoprecipitate must normally be diluted with at least 0.5 parts of an aqueous solution in order to reduce its viscosity for further processing.

20 The present inventor has found that by adding at least 0.15mg of an SPS (preferably heparin) per ml of cryoprecipitate solution, the SPS acts as a selective precipitant for fibrinogen and fibronectin whilst leaving a high proportion of the factor VIII intact in solution. Both the degree of precipitation and the yield of factor
25 VIII left in solution are found to be generally less dependent upon citrate anti-coagulant concentration in the blood plasma fraction than the method of Bier et al. Furthermore, factor VIII yield appears to be less dependent upon precipitant concentration than the method of Bier et al. Although useful precipitation of fibrinogen and fibroⁿectin is achieved at as little as 0.15 mg heparin/ml of cryoprecipitate solution, more efficient removal of fibrogen and fibronectin can be gained by adding up to 1.2 mg heparin/ml without commensurate losses of Factor VIII into the precipitate. Heparin is
30 preferably added to the cryoprecipitate solution to a concentration of 0.3 mg/ml to 0.9 mg/ml.

35 Another significant advantage of the present invention is that provided a sufficiently high concentration of the SPS is used,

precipitation of fibrinogen is maximal after about 5 minutes mixing and no significant further precipitation of factor VIII occurs up to at least 20 minutes. Short reaction times and tolerance of extended holding periods are advantages for large-scale processing.

5 Factor VIII has a relatively narrow range of pH stability. It is most stable in the neutral pH range of 6.0 to 8.0, hence the pH of the blood plasma fraction is preferably within this range both during and after SPS addition. Blood plasma fraction pH may be adjusted if necessary using an appropriate buffer solution. Most
10 preferably, however, the pH of the fraction is kept within the range 6.0 to 7.0. Above pH 7.0, useful precipitation of fibrinogen and fibronectin is obtained only at very high SPS concentrations. As pH decreases from pH 7 to pH 6, factor VIII losses into the precipitate increase as precipitation of fibrinogen and fibronectin increase.
15 pH can therefore be easily manipulated by, for example, the use of buffer solutions, to give a range of compromises between factor VIII yield and purity required.

The plasma fraction is preferably maintained within the temperature range 20°C to 35°C during precipitation. Within this temperature range, precipitation is rapid and Factor VIII losses are low.
20 Most preferably, the concentrate is maintained within the temperature range 25°C to 30°C.

After SPS precipitation has been completed, the precipitate is preferably removed without delay from the supernatant by, for
25 example, centrifugation. The precipitate may be discarded or may be further processed to extract the fibronectin (which is becoming of increasing clinical interest) and the fibrinogen.

SPS precipitation may be used alone to purify a blood plasma fraction or may be used in conjunction with other purification steps
30 as part of a factor VIII purification process.

Where purification consists solely of SPS precipitation, then residual SPS remaining in the supernatant after precipitation may be left in solution or may be removed in part or in full by, for example, the conventional step of adsorption onto $Al(OH)_3$ hydrogel.
35 It is one advantage of the present invention where the SPS consists of heparin that complete removal of heparin is normally unnecessary, because heparin levels of about 1 unit/ml (0.06 mg/ml) of

cryoprecipitate solution are, in accordance with the teachings of Fekete et al (US Patent No 3,803,115) both safe and beneficial to factor VIII stability. The preparation may then be stabilised and further concentrated by freeze drying, followed by reconstitution in aqueous solution to an appropriate concentration before use.

Where SPS precipitation forms only part of a factor VIII purification process, then this precipitation can be carried out after certain other purification steps. Preferably, however, SPS precipitation is carried out as early as possible in the process, because fibronectin and especially fibrinogen are known to influence other purification steps and so efficient early removal of these contaminants is desirable.

In a commercial factor VIII purification process, SPS precipitation will normally be followed by one or more known concentration steps optionally in conjunction with a pasteurisation step.

Pasteurisation is an especially important step because it inactivates potentially harmful viruses which are transmissible by blood (eg hepatitis viruses) and which are carried over into plasma fraction) such as cryoprecipitate. A typical pasteurisation step consists of heat treatment in solution at 60°C for 10 hours. Large quantities of carbohydrate eg sorbitol and of amino-acids such as glycine are usually added to help stabilise factor VIII at these temperatures, and factor VIII yields through pasteurisation are also improved by the addition of small amounts of citrate ions and calcium ions. The presence of residual SPS from the earlier SPS precipitation step is not found to have any significant effect on factor VIII yields through a typical pasteurisation step. After heating, the factor VIII can be recovered and concentrated by ultrafiltration or by precipitation with glycine and sodium chloride, followed by desalting.

Two other known methods of preparing "heat-treated" concentrates of factor VIII, with the intention of inactivating blood-borne viruses, have been found to be most successful after efficient removal of fibrinogen and fibronectin by precipitation with heparin.

(a) Factor VIII is precipitated from the heparin-containing supernatant by addition of high concentrations of glycine and sodium chloride, and the precipitate redissolved in a small

volume of buffer solution. After desalting by gel filtration, the solution is sterilised, freeze-dried and heated in its final container to temperatures of at least 70°C for at least 24h with little or no loss of factor VIII activity or solubility.

(b) The factor VIII, reprecipitated and redissolved as in (a), is pasteurised in solution after the addition of high concentrations of sorbitol and glycine. The heated factor VIII is then recovered by ultrafiltration or by a second precipitation with glycine and sodium chloride, followed by desalting.

Methods of precipitating fibrinogen and fibronectin from blood plasma concentrates in accordance with the present invention will now be described by way of example only.

MATERIALS

The following materials were used in the Examples

1. Heparin.

The heparin used was porcine intestinal mucosal heparin in the form of US Pharmacopoeia (USP) Grade 1 sodium heparin supplied by Sigma Chemical Co, St Louis USA having a specific activity of about 168 units (u) per milligram. The heparin was made up into a standard stock solution having a heparin concentration of 3750 u/ml (22 mg/ml). Other sources of heparin USP may also be used.

METHODS

The following methods were used in the Examples

1. Method of preparing cryoprecipitate

Whole blood anticoagulated with sodium citrate anticoagulant was centrifuged within a few hours of donation and the separated plasma frozen to -25°C to -40°C in plastic containers. Before cryoprecipitation proper, the frozen plasma was softened to -5°C to -15°C by storage for several hours in a room at 0-5°C, and the thawing process was facilitated by breaking up the frozen plasma. The pieces of frozen plasma were warmed in a jacketed vessel under efficient manual or mechanical stirring, so that no part of the suspension rose above a temperature of about +3°C to prevent the desired cryoprecipitate from redissolving in the liquid phase. Ideally, the suspension was kept at +1°C. The cryoprecipitate was separated from the partly thawed suspension by continuous centri-

fugation in a Sharples continuous tubular centrifuge maintained at about +1°C to +3°C. The cryoprecipitate was redissolved by mixing at 20°C-25°C with a volume of 20mM tris-HCl buffer, pH 6.8, equivalent to 2.4% of the volume of the plasma taken, and the solution was used fresh or was stored frozen at -30°C for later use.

2. Method of precipitating fibrinogen and fibronectin using heparin

Cryoprecipitate solution was thawed to about 25°C and was taken either undiluted or, in some Examples, diluted with up to one volume of 20 mM tris pH 6.8. The pH of the thawed cryoprecipitate was adjusted to a desired level if necessary by mixing in small quantities of either 0.1M or 0.05M HCL. Stock heparin solution was then added in a rapid stream by pipette or syringe whilst continuously stirring the mixture. After thorough mixing the suspension containing fibrinogen and fibronectin precipitate was immediately centrifuged at 4000 xg for 10 minutes, and the supernatant poured off. All steps were performed at about 25°C.

ASSAYS

The following assay techniques were employed on the blood plasma concentrates both before and after conducting the methods described in the Examples, in order to determine yields of various components in the concentrates.

1. Factor VIII

Two-stage Factor VIII Coagulant (factor VIIIIC) assays were done usually on frozen but occasionally on fresh samples. Heparin-containing samples were routinely absorbed with 0.1 volume of Al(OH)₃ (Al hydrogel) for 3 minutes at 37°C in order to remove the heparin before assay.

2. Total protein and fibrogen

Total protein was assayed by the biuret method. Fibrogen was measured as the component of the protein which was clottable with thrombin.

3. Fibronectin

Fibronectin was assayed by Laurell immunoelectrophoresis.

4. Heparin

Heparin was assayed by the inhibition of factor Xa activity, measured by the rate of hydrolysis of the synthetic substrate S-2222

(Tein A N, Lie, M and Abildgaard, U. Thrombosis Research, 8, 413, 1976).

Examples 1-7

Heparin was added to a final concentration of 0.31 or 0.44 mg/ml to undiluted aliquots of the same cryoprecipitate solution, adjusted to pH values between 5.8 and 7.1. The resulting mixtures were centrifuged after five minutes. The results of heparin precipitation in terms of yields of factor VIII:C, fibrinogen and fibronectin in the supernatant are given in Table 1 below.

10 Table 1

Example	Heparin added mg/ml	Supernatant pH	%yields in supernatant		
			Factor VIII:C	Fibrinogen	Fibronectin
1	0.44	5.8	42	10	0
2	0.44	6.4	65	7	15
3	0.44	6.5	70	8	16
4	0.44	6.6	68	12	20
5	0.44	6.7	66	15	27
6	0.31	6.5	69	11	42
7	0.31	7.1	72	30	74

From these and similar experiments at other pH values and heparin concentrations, it was established that the precipitation of fibrinogen and fibronectin tended to increase as pH was reduced, at the cost of increasing loss of factor VIII, with a preferred compromise at about pH 6.5.

Examples 8 to 13

Samples of between 5 ml and 1500 ml of thawed cryoprecipitate solution extracted from various blood plasma sources were used, some of which were diluted with one volume of 0.02M tris pH 6.8. pH was adjusted to 6.55 ± 0.03 by the addition of HCl solution. Heparin was then admixed to each sample to a concentration of between 0.22 mg/ml and 0.88 mg/ml to effect fibronectin and fibrinogen precipit-

ation. The resulting mixture was centrifuged after 5 minutes. The results of heparin precipitation in terms of yields of factor VIIIIC, fibrinogen, and fibronectin are given in Table 2 below.

Table 2 (Effect of cryoprecipitate dilution)

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Example	Volume of 0.02M tris added	Heparin added mg/ml	Number of experiments performed n	% yields in supernatant*		
				F VIII:C	Fibronegen	Fibronectin
8	0	0.22	17	78 ₊₇	19 ₊₃	51 ₊₁₂
9	0	0.44	9	75 ₊₁₁	10 ₊₃	29 ₊₆
10	0	0.88	1	56	4	11
11	1	0.22	6	75 ₊₆	7 ₊₂	28 ₊₃
12	1	0.44	1	68	6	31
13	1	0.67	1	65	9	35

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*standard deviations from mean yields are given where n > 1.

20 The above Table 2 shows that fibrinogen and fibronectin removal increase with increasing heparin concentration. Dilution of cryoprecipitate solution with tris buffer (ie reduction of protein concentration) permits more efficient removal of fibrinogen and fibronectin, with good factor VIII yield, at lower heparin concentrations. On an industrial scale, however, it is preferable to avoid dilution of the cryoprecipitate solution and to use a heparin concentration of about 0.66 mg/ml, giving excellent separation of factor VIII from fibrinogen and fibronectin similar to that achieved in diluted solution at 0.44 mg heparin/ml.

30 Examples 14 to 18

10 ml samples of cryoprecipitate solution, each taken from the same blood plasma source, were taken and the pH of each sample was adjusted to pH 6.55 ± 0.03 by the addition of HCl solution. Heparin was then admixed to each sample to a concentration of between 0.22 mg/ml and 0.88 mg/ml to effect fibronectin and fibrinogen precipitation. The resulting mixture was centrifuged after 5 minutes. The results of heparin precipitation in terms of yields of factor VIIIIC,

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fibrinogen, and fibronectin are given in Table 3 below.

Table 3 (Effect of heparin concentration)

Example	Heparin added mg/ml	%yields in supernatant		
		Factor VIII:C	Fibrinogen	Fibronectin
14	0.22	67	29	37
15	0.33	68	16	20
16	0.44	69	11	16
17	0.66	75	6	20
18	0.88	67	3	13

The above Table 3 shows that, with increasing heparin concentration, precipitation of fibrinogen and fibronectin tends to increase but the yield of Factor VIII remains relatively constant. Examples 16 (Comparative)

The method of Example 6 was repeated on nine separate samples of thawed cryoprecipitate using zinc ions at a concentration of 1.5 mM in the cryoprecipitate instead of heparin, producing yields (\pm standard deviations) of $71\pm 9\%$ factor VIIIIC, $24\pm 5\%$ fibrinogen, and $51\pm 8\%$ fibronectin in the supernatant. These results compare unfavourably with those of Examples 2 to 6 above both in terms of factor VIIIIC yield and in terms of the quantities of fibrinogen and fibronectin precipitated. The high levels of fibrinogen and fibronectin left in solution have a marked influence on the properties, especially the solubility, of the concentrates obtained by further processing eg pasteurisation or heating in the freeze-dried state.