A protocol for the preparation of cryoprecipitate and cryo-depleted plasma for proteomic studies

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Running Title: cryoprecipitate and cryo-depleted plasma protocol

Key Words: plasma, cryoprecipitate, anti-hemophilic factor, factor VIII, cryo-depleted, fibrinogen, proteomics, cryosupernatant

Abstract

Cryoprecipitate is a concentrate of high-molecular weight plasma proteins that precipitate when frozen plasma is slowly thawed at 1-6 °C. The concentrate contains Factor VIII (anti-hemophilic factor), von Willebrand factor (vWF), fibrinogen, Factor XIII, fibronectin and small amounts of other plasma proteins. Clinical grade preparations of cryoprecipitate are mainly used to treat fibrinogen deficiency caused by acute bleeding or functional abnormalities of the fibrinogen protein. In the past cryoprecipitate was used to treat von Willebrand disease and hemophilia A (Factor VIII deficiency), but the availability of more highly purified coagulation factor concentrates or recombinant protein preparations has superseded the use of cryoprecipitate for these coagulopathies. Cryo-depleted plasma ('cryosupernatant') is the plasma supernatant remaining following removal of the cryoprecipitate from frozen-thawed plasma. It contains all the other plasma proteins and clotting factors present in plasma that remain soluble during cold-temperature thawing of the plasma. This protocol describes the clinical-scale preparation of cryoprecipitate and cryo-depleted plasma for proteomic studies.

1. Introduction

Cryoprecipitate (also known as cryoprecipitated anti-hemophilic factor) was first described in the mid-1960s as a method to concentrate factor VIII (anti-hemophilic factor) from plasma for use in patients with hemophilia, von Willebrand disease or hypofibrinogenemia [1, 2]. Cryoprecipitate is the insoluble concentrate of high-molecular weight plasma proteins that precipitate when frozen plasma is slowly thawed at 1-6 °C [2]. Cryoprecipitate is enriched for plasma coagulation proteins, in particular factor VIII, fibrinogen, von Willebrand factor, factor XIII and fibronectin. Small amounts of other plasma proteins, such as immunoglobulins, may also be present.

Cryoprecipitate is a standard blood transfusion component manufactured by most blood transfusion services [3]. Clinically, the main indications for use of cryoprecipitate is for the treatment of fibrinogen deficiency (hypofibrogenemia), caused by significant bleeding due to trauma, massive transfusion or disseminated intravascular coagulation, or dysfibrogenemia arising from functional abnormal fibrinogen [3, 4]. Previously, cryoprecipitate was used to treat hemophilia and von Willebrand disease, but with the advent of specific coagulation factor concentrates, it has been relegated to second-line therapy for these diseases. Over the years attempts have been made to improve the yield in cryoprecipitate by the use of techniques such as thaw-siphon [5], the effect of temperature freezing and thawing [6], or by the use of various additives, such as heparin [7] and sodium citrate [8, 9].

Cryo-depleted plasma (cryosupernatant) is the plasma supernatant remaining following removal of the precipitated cold insoluble proteins (i.e. cryoprecipitate) from frozen-thawed plasma. It is therefore significantly depleted of fibrinogen, factor VIII, von Willebrand

factor, factor XIII and fibronectin, but contains all the other plasma proteins and clotting factors in similar concentrations as the original plasma. Clinically cryo-depleted plasma is used for plasma exchange in thrombotic thrombocytopenic purpura and in situations requiring rapid temporary reversal of warfarin anti-coagulant therapy [10].

In this protocol, the preparation of small research-scale cryoprecipitate and cryo-depleted plasma is outlined, and is based on the procedures used by blood transfusion services for the preparation of clinical-scale cryoprecipitate [10].

2. Materials

2.1 Blood collection, plasma preparation and storage

1. Whole blood collection tubes containing citrate anticoagulant (e.g. BD Vacutainer 4.5 mL tube with 0.5 mL 3.2% sodium citrate anticoagulant, BD Biosciences #366415; BD Vacutainer 8.5 mL tube with 1.5 mL acid citrate dextrose (ACD) Sol A anticoagulant (22.0 g/L trisodium citrate, 8.0 g/L citric acid, and 24.5 g/L dextrose), BD Biosciences #364606) (**Note 1**)
2. Blood collection needles compatible with the blood collection tubes e.g. BD Vacutainer® Safety-Lok™ Blood Collection Set #367283; 23G butterfly needle with attached sterile tubing)
3. Alcohol and swabs for disinfection of the venipuncture site
4. Personal protective equipment, gloves, gown, eye safety glasses
5. Disposal container for biological hazards
6. Polypropylene tubes (1.5 mL, 15 mL)
7. Labels for blood sample tubes

8. Tube storage rack
9. Centrifugation unit (with swing-bucket rotor, compatible with 1.5/15 mL tubes, programmable temperature setting; range 4-25 °C)
10. Pipettes
11. Freezer (-20 °C or lower)

2.2 Cryoprecipitate/cryo-depleted plasma preparation

1. Refrigerator or water bath set at 4 ± 2 °C
2. Centrifuge, refrigerated (swing bucket rotor, compatible with 1.5/15 mL tubes, programmable temperature setting)
3. 0.9% saline solution

3. Methods

3.1 Blood collection/phlebotomy

1. Blood collection must only be performed by personnel trained in phlebotomy/venipuncture. Safety precautions for the collection and handling of blood must be employed at all times (**Note 2**). Particular care must be taken with insertion of the needle into the vein to limit the possibility of activation of the coagulation factors in the blood, which could compromise the quality of the blood sample.
2. It is important to collect the volume of blood specified for the particular type of blood collection tube to ensure the correct blood/anticoagulant ratio is achieved (**Note 3**).
3. After blood collection, gently mix the blood by inverting the tube several times to ensure thorough mixing with the anti-coagulant. For thorough mixing of blood collected into citrate tubes, it is recommended to invert the tube 3-4 times, whilst ACD tubes should be inverted 8 times.
4. Blood samples should be maintained at temperate conditions (i.e. 20-24 °C) and centrifuged within four hours of blood collection. Superior factor VIII yields are obtained from blood that is maintained at 20-24 °C before processing.
5. To separate the plasma, centrifuge the blood samples at 1,200 × *g* for 10 min at 22 °C. If needed, RCF for a centrifuge can be calculated. For an on-line calculator tool, refer: http://www.currentprotocols.com/tools/g-forcerpm-conversion-tool
6. After centrifugation, the plasma layer will be the upper layer of the separated blood and the cellular fractions are the lower layers. The plasma should be a clear, straw-yellow colored fluid (**Note 4**). Mononuclear cells and platelets form a thin whitish layer (buffy coat) that settles directly on top of the red blood cell layer.

7. Carefully collect the plasma layer with an appropriate transfer pipette without disturbing the buffy coat layer. Do not attempt to collect all the plasma. Do not allow the tip of the transfer pipette within 5 mm of the buffy coat layer and avoid touching the wall of the tube with the pipette. This helps to avoid inadvertent contamination of the plasma with cells that may only be softly sedimented in the buffy coat-plasma interface (**Note 4**). If more than one tube of blood is collected from the same donor, pool the plasma samples from both tubes into a 15 mL polypropylene tube. If necessary, aliquot plasma into smaller volumes. A practical minimum volume is 1-1.5 mL.
8. Close the tube tightly and place on ice or immediately freeze by placing in the freezer. This process should be completed within 30 min of centrifugation. Plasma should be frozen as quickly as possible to minimize loss of labile coagulation factors, such as Factor VIII. Frozen plasma should be stored at below -20 °C.

3.2 Cryoprecipitation

1. For the preparation of cryoprecipitate, remove tube(s) of frozen plasma from the freezer and immediately place in a thermostatically-controlled water bath or refrigerator set at 1-6 °C. Slowly thaw the plasma until it becomes "slushy" (required time will depend on volume of plasma being thawed) (**Note 5**). Optimum temperature for cryoprecipitate formation is 3 °C.
2. Immediately sediment precipitated proteins in a refrigerated centrifuge (1-6 °C) at 5,000 × g for 15 min. A white precipitate should be evident in the bottom of the tube.
3. Carefully remove the supernatant (Note: this is the cryo-depleted plasma). If this cryo-depleted plasma is required, aliquot into separate polypropylene tube(s). Leave a small amount of plasma above the deposited cryoprecipitate (5-10% v/v, 50-100 μL

for 9-10 mL blood collection volume). If the cryoprecipitate or cryo-depleted plasma is not required immediately, freeze at -20 °C (**Note 6**).

3.3 Thawing of cryoprecipitate/cryo-depleted plasma

1. Thaw cryoprecipitate or cryo-depleted plasma in a water bath at 30-37 °C. The cryoprecipitate should be evenly dissolved at warming temperature.
2. Cryoprecipitate can be suspended in diluent, such as 0.9% saline, at 20–24 °C. Cryoprecipitate should be maintained at 20-24 °C and used within 6 h of thawing. Progressive functional decline of labile proteins, such as Factor VIII, occurs following thawing (**Notes 7-10**).
3. Thawed cryo-depleted plasma can be maintained at 2-6 °C.

4. Notes

1. **Anticoagulant.** For all physiological coagulation studies sodium citrate or ACD are the anticoagulants of choice. Cryoprecipitate prepared by blood transfusion services for clinical use is prepared from whole blood collected into citrate-phosphate-dextrose (CPD) anticoagulant or from plasma collected into ACD anticoagulant by apheresis [11]. Cryoprecipitate prepared from CPD anticoagulated plasma has been shown to give a higher yield of Factor VIII compared to ACD anticoagulated plasma [11]. The preparation of cryoprecipitate from plasma anticoagulated with non-citrate anticoagulants (e.g. heparin) may yield a different profile of precipitated proteins [12].

2. **Safety.** All blood and biological specimens and materials coming in contact are considered biohazards. Use gloves, gowns, eye protection, other personal protective equipment, and controls to protect from blood splatter, blood leakage, and potential exposure to blood borne pathogens. Use aseptic technique and sterile disposables (tubes, pipettes) throughout to prevent contamination of the blood. Risk factors for possible transfusion transmissible infections should be rigorously screened for prior to blood collection. Handle as if capable of transmitting infection and dispose of with proper precautions in accordance with federal, state and local regulations. Refer to your institutional regulations regarding the screening of blood for specific infectious disease markers (i.e. HIV, Hepatitis B, Hepatitis C, etc). Discard all blood collection materials in biohazard containers approved for their disposal.

3. **Evacuated blood collection tubes** (e.g. BD Vacutainers) are manufactured to draw the blood volume specified for the particular tube. Filling is complete when vacuum

no longer continues to draw blood into the tube. Partially filled tubes will not have the correct blood/anticoagulant ratio and should not be used for the purpose of plasma sample preparation. If a citrated blood sample is the first sample to be collected from the donor it is important to first collect a small volume of blood into a discard tube. This ensures that the "dead" volume in the needle/tubing set is filled with blood prior to the citrated tube being connected, thus ensuring the correct blood volume is drawn into the tube.

4. **Quality control of plasma sample.** It is recommended that following the preparation of the plasma sample that the quality of the plasma is monitored for hemolysis, clarity and contamination with cells and/or platelets. Manual or automated cell counting methods should be validated by your institution or testing laboratory. Normal plasma should be straw-yellow in colour and the clarity should be relatively clear. Plasma from female donors who are taking oral contraceptives can have a green coloration and is considered normal [13]. Pink or red coloration of the plasma is indicative of hemolysis of red blood cells and is not normal [14]. Hemolysis could be due to poor collection or handling of the blood sample or due to donor-related medical factors. Milky opaque plasma is due to raised lipid content [15]. Plasma lipids can be transiently raised in a healthy individual due to the recent consumption of a high fat meal (postprandial-induced lipemia). Except in the instance of postprandial-induced lipemia, very opaque plasma is not normal. For protein chemistry studies of normal plasma, samples with high lipid content or hemolysis should be avoided. Plasma that has been previously thawed and refrozen will give inferior yields if used for the preparation of cryoprecipitate.

5. **How to prevent cryoprecipitation when thawing plasma.** If plasma is to be used for coagulation studies, it is important to prevent cryoprecipitation from occurring. To avoid cryoprecipitation of the cold-insoluble proteins when thawing replete plasma, plasma must be thawed quickly at 37 °C. This can be achieved by placing the frozen plasma samples in a 37 °C water bath or dry heating system set at 37 °C. Such equipment must be maintained and kept clean to avoid inadvertent bacterial contamination of plasma samples.

6. **Cryoprecipitate and cryo-depleted plasma storage.** The prepared cryoprecipitate and cryo-depleted plasma can be used up to 12 months when stored at or below -18 °C [3] . When cryoprecipitate is thawed and held at 20-24 °C, the functional levels of non-labile proteins, such as fibrinogen and Factor XIII, remain stable for up to 72 h, while labile proteins such as Factor VIII decline within 24 h [16].

7. **Cryoprecipitate content and specifications.** According to blood transfusion guidelines, a unit of cryoprecipitate prepared from the plasma of a standard 450-500 mL CPD-anticoagulated whole blood donation should contain at least 150 mg of fibrinogen and a minimum of 80 international units (IU) of Factor VIII [3, 10, 11, 17]. This equates to 30-70% of the Factor VIII/vWF and fibrinogen content of the original plasma. Proportionally similar yields should be achieved when cryoprecipitate is prepared from smaller starting volumes of plasma providing care is taken with the processing, freezing and thawing of the plasma samples as described in this protocol.

8. **Effect of ABO blood group.** Plasma/cryoprecipitate from blood group O individuals has lower levels of Factor VIII and vWF than A, B or AB blood groups.

9. **Effect of additional treatment steps on coagulation factor proteins**. Some blood transfusion services supply plasma units that have undergone additional treatment steps to further minimize the already very low risk of infectious disease transmission or adverse reactions by blood transfusion. Various pathogen reduction technologies have been developed, including treatments with solvent/detergent, or photoactivating agents such as methylene blue, riboflavin or psoralen [18]. Removal of leukocytes by filtration before processing of whole blood is commonly performed by blood transfusion services. A trade-off for the increased safety rendered by these treatments is a reduced yield and activity of plasma coagulation factors, including fibrinogen and Factor VIII [18-20]. The nature of any biochemical changes that occur to the coagulation proteins following pathogen reduction treatment is yet to be fully determined.

10. **Extracellular vesicles.** Cryoprecipitate has been reported to contain a significant enrichment of extracellular vesicles (also called microparticles or microvesicles) present in normal plasma [21] (Chan & Sparrow, unpublished findings). Moreover, high levels of extracellular vesicles in cryoprecipitate may contribute to its therapeutic effects in bleeding patients [21]. It is yet to be determined whether extracellular vesicles in cryoprecipitate are biologically functional, such as in hemostasis, inflammation and/or allo-immunoreactivity [22].

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