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(54) **METHOD OF PURIFYING THERAPEUTIC PROTEINS**

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USPC **424/94.64**; 530/382; 514/13.7

(57) **ABSTRACT**

The present invention relates generally to a method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or von Willebrand factor (VWF), the method comprising: (i) passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a hydrophobic charge-induction chromatographic resin under conditions selected such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the resin; and (ii) recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the resin; wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or protease(s) in the recovered solution is reduced by at least 50% compared to the feedstock. Also provided are solutions and pharmaceutical formulations comprising the fibrinogen and/or Factor VIII and/or VWF recovered by such methods, and uses thereof.

FIGURE 1

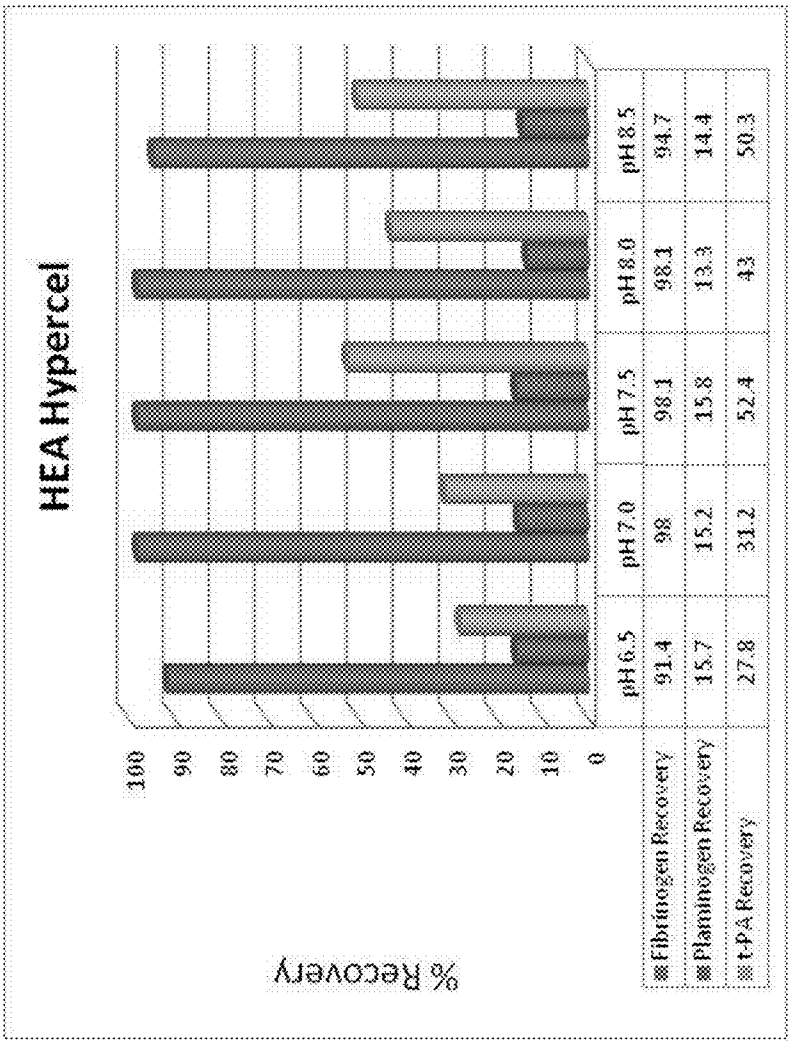

HEA Hypercel
% Recovery
100
90
80
70
60
50
40
30
20
10
0
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
Fibrinogen Recovery
91.4
98
98.1
98.1
94.7
Plasminogen Recovery
15.7
15.2
15.8
13.3
14.4
t-PA Recovery
27.8
31.2
52.4
43
50.3

FIGURE 2

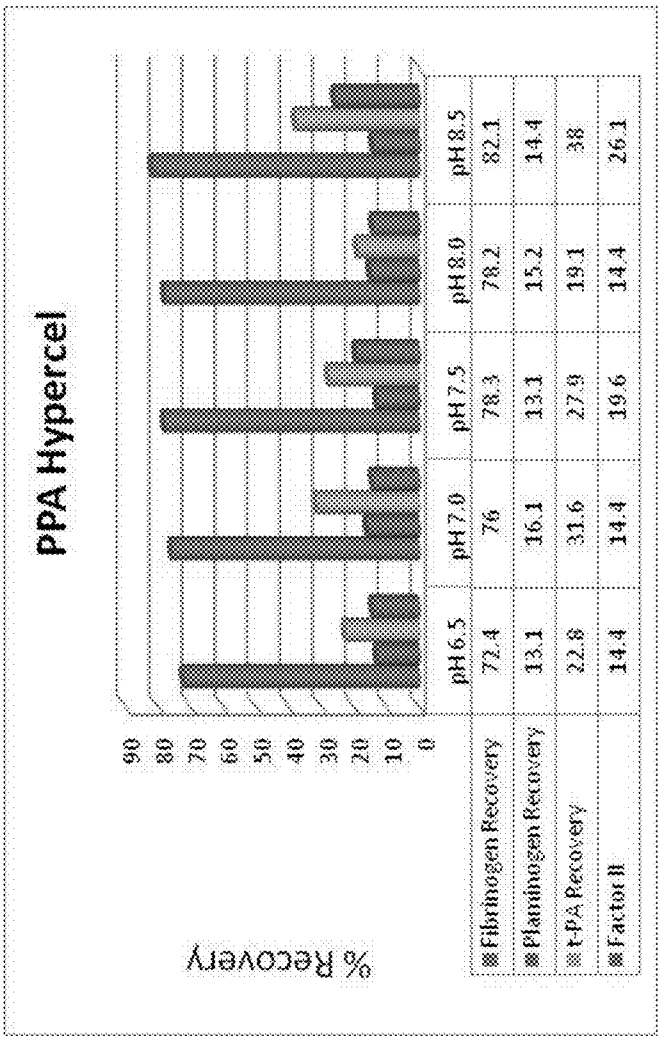

PPA Hypercel
% Recovery
90
80
70
60
50
40
30
20
10
0
pH6.5
pH7.0
pH7.5
pH8.0
pH8.5
Fibrinogen Recovery 72.4 76 78.3 78.2 82.1
Plasminogen Recovery 13.1 16.1 13.1 15.2 14.4
t-PA Recovery 22.8 31.6 27.9 19.1 38
Factor II 14.4 14.4 19.6 14.4 26.1

FIGURE 3

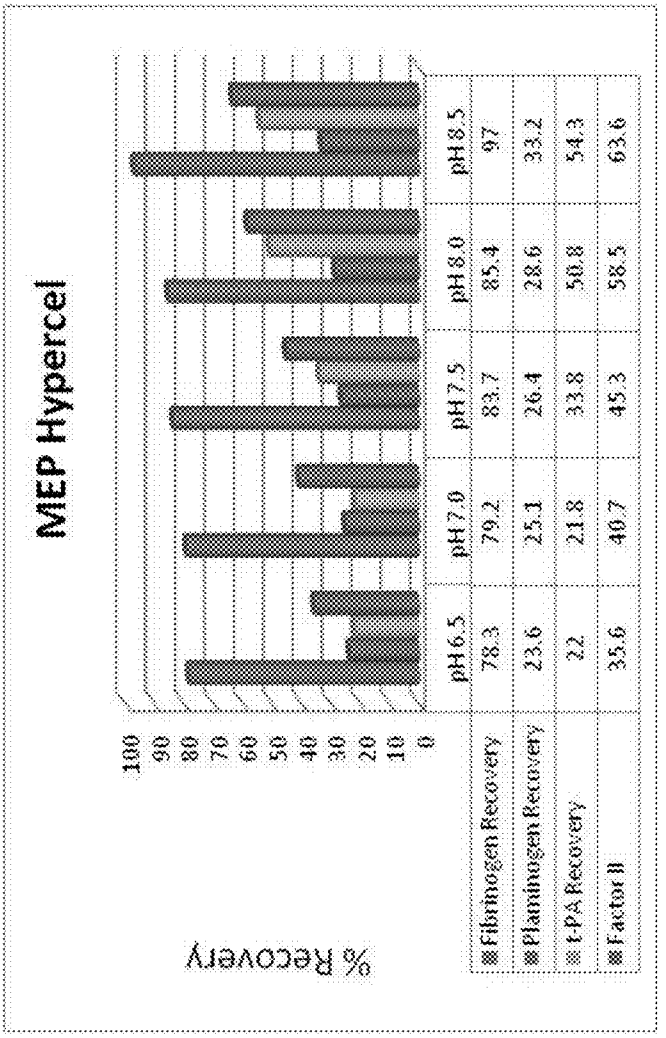

MEP Hypercel
% Recovery
100
90
80
70
60
50
40
30
20
10
0
pH6.5
pH7.0
pH7.5
pH8.0
pH8.5
Fibrinogen Recovery 78.3 79.2 83.7 85.4 97
Plasminogen Recovery 23.6 25.1 26.4 28.6 33.2
t-PA Recovery 22 21.8 33.8 50.8 54.3
Factor II 35.6 40.7 45.3 58.5 63.6

FIGURE 4A

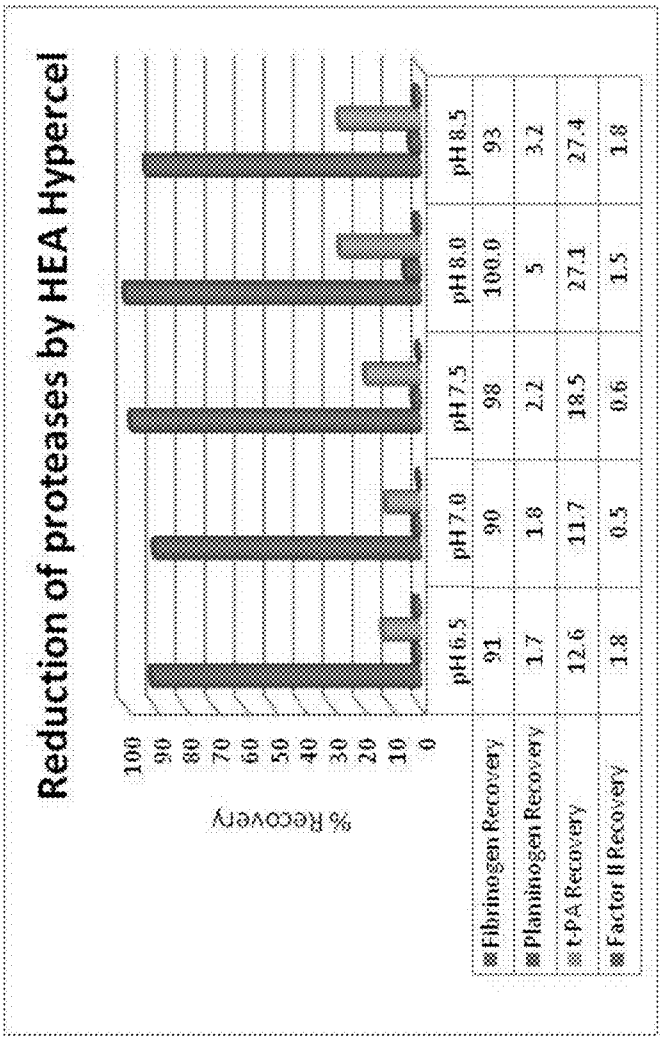

Reduction of proteases by HEA Hypercel
% Recovery
100
90
80
70
60
50
40
30
20
10
0
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 8.5
Fibrinogen Recovery 91 90 98 100.0 93
Plasminogen Recovery 1.7 1.8 2.2 5 3.2
t-PA Recovery 12.6 11.7 18.5 27.1 27.4
Factor II Recovery 1.8 0.5 0.6 1.5 1.8

FIGURE 4B

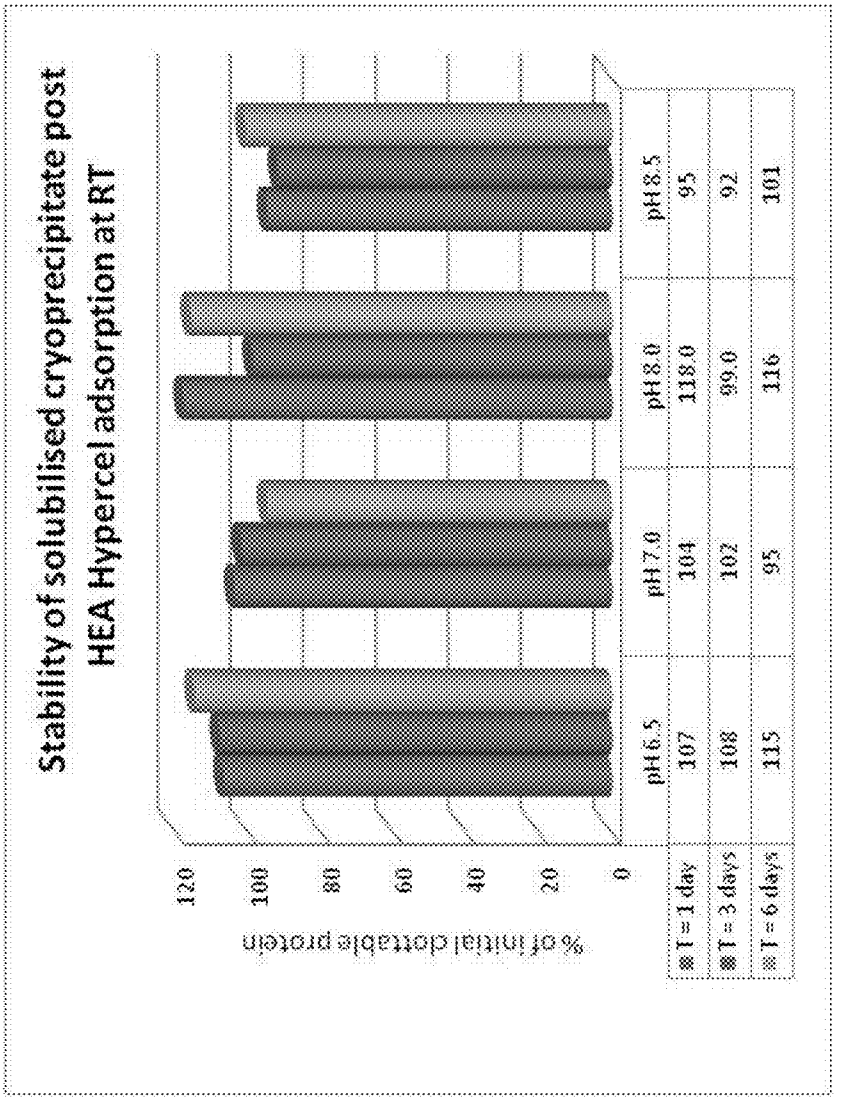

Stability of solubilised cryoprecipitate post HEA Hypercel adsorption at RT
% of initial clottable protein
120
100
80
60
40
20
0
pH6.5
pH7.0
pH8.0
pH8.5
T = 1 day 107 104 118.0 95
T = 3 days 108 102 99.0 92
T = 6 days 115 95 116 101

FIGURE 4C

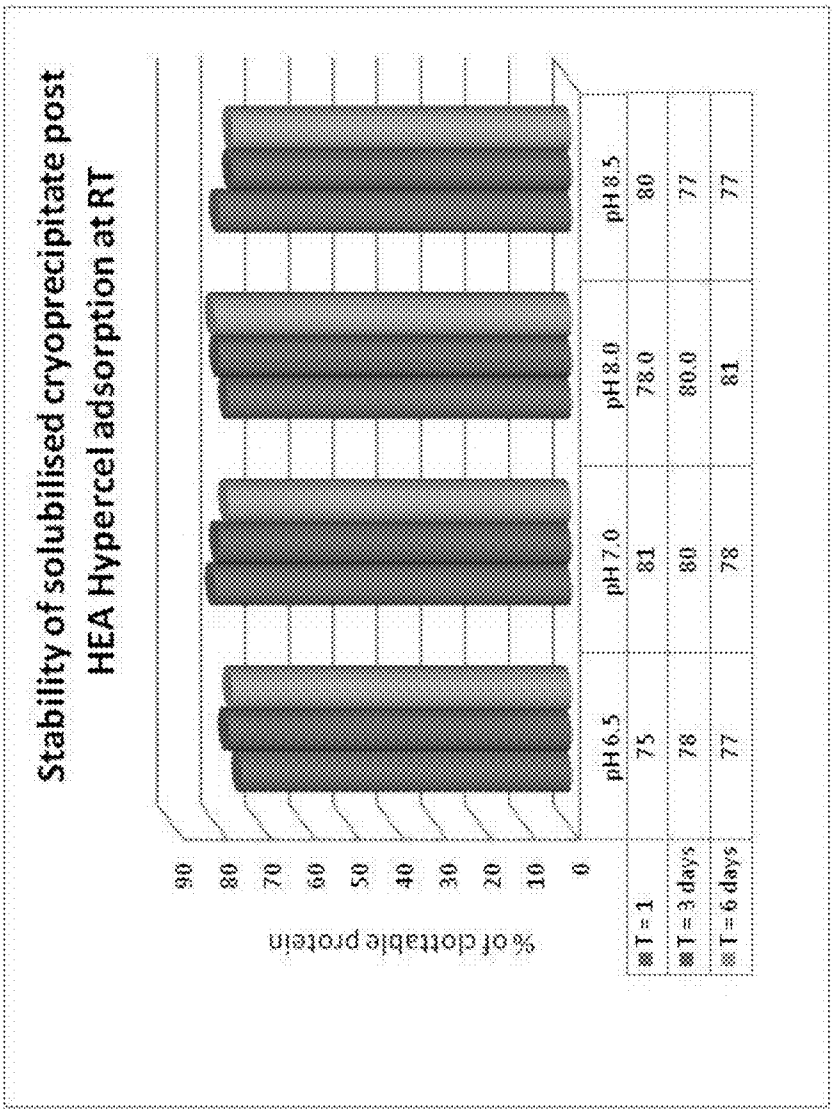

Stability of solubilised cryoprecipitate post HEA Hypercel adsorption at RT
% of clottable protein
90
80
70
60
50
40
30
20
10
0
pH6.5
pH 7.0
pH8.0
pH8.5
T = 1
75
81
78.0
80
T = 3 days
78
80
80.0
77
T = 6 days
77
78
81
77

FIGURE 5

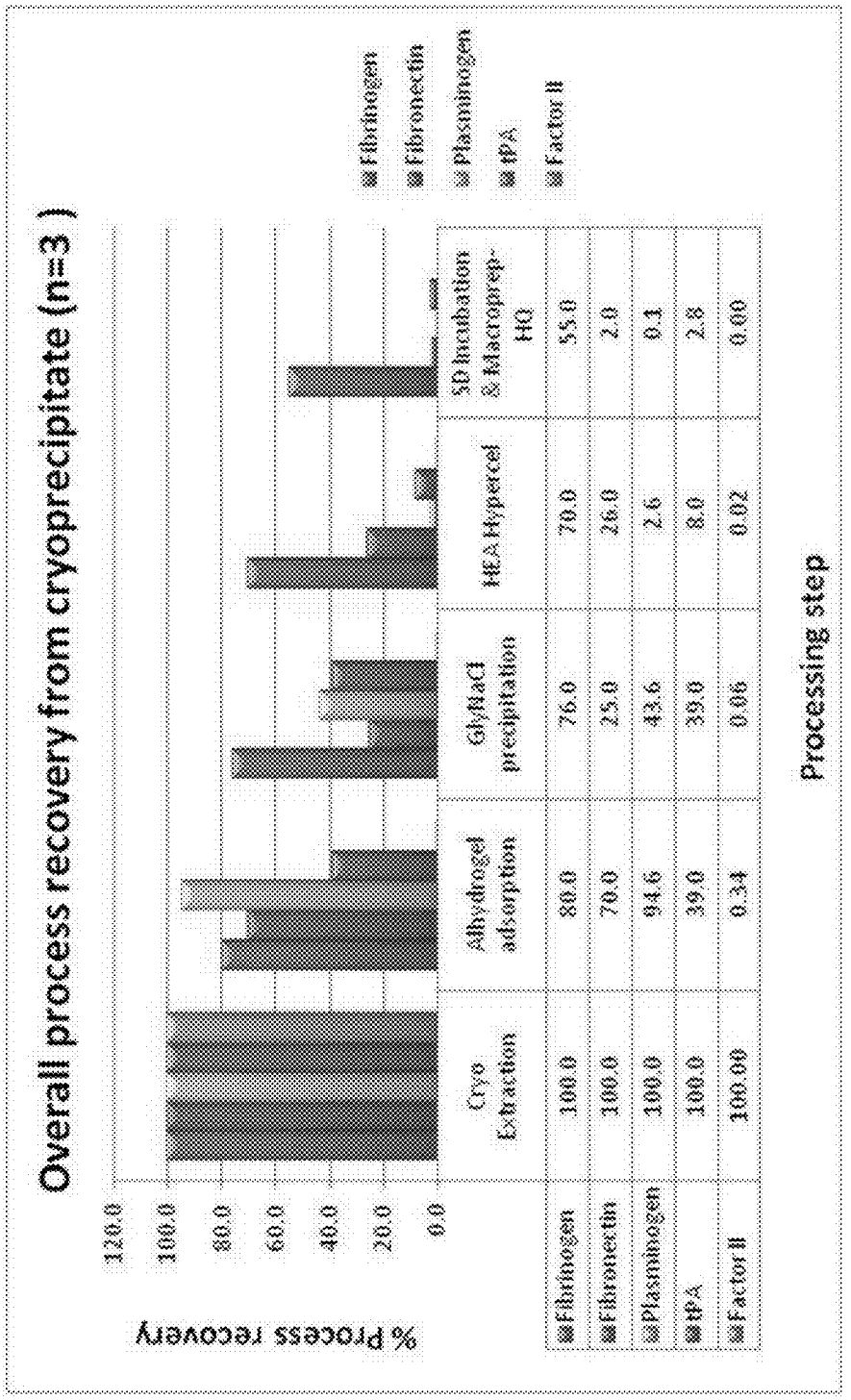
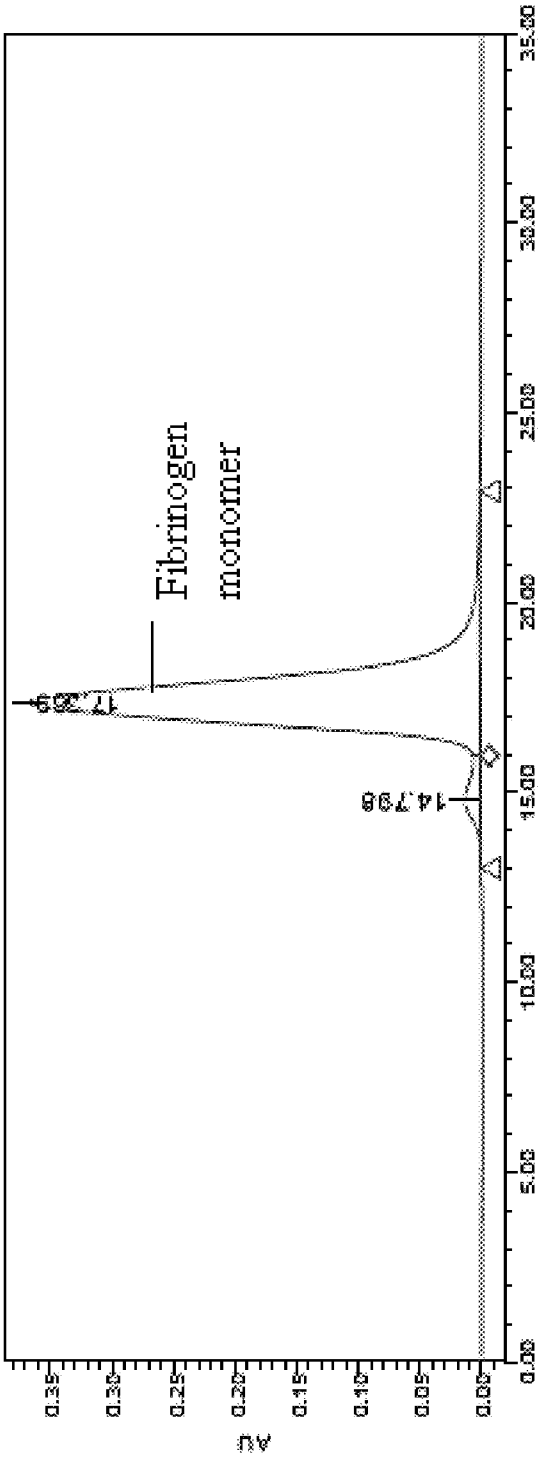

Overall process recovery from cryoprecipitate (n=3)
% Process recovery
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Fibrinogen
Fibronectin
Plasminogen
tPA
Factor II
Processing step
Cryo Extraction
Alhydrogel adsorption
Gly/NaCl precipitation
HEA Hypercel
SD Incubation & Macroprep-HQ
Fibrinogen 100.0 80.0 76.0 70.0 55.0
Fibronectin 100.0 70.0 25.0 26.0 2.0
Plasminogen 100.0 94.6 43.6 2.6 0.1
tPA 100.0 39.0 39.0 8.0 2.8
Factor II 100.00 0.34 0.06 0.02 0.00

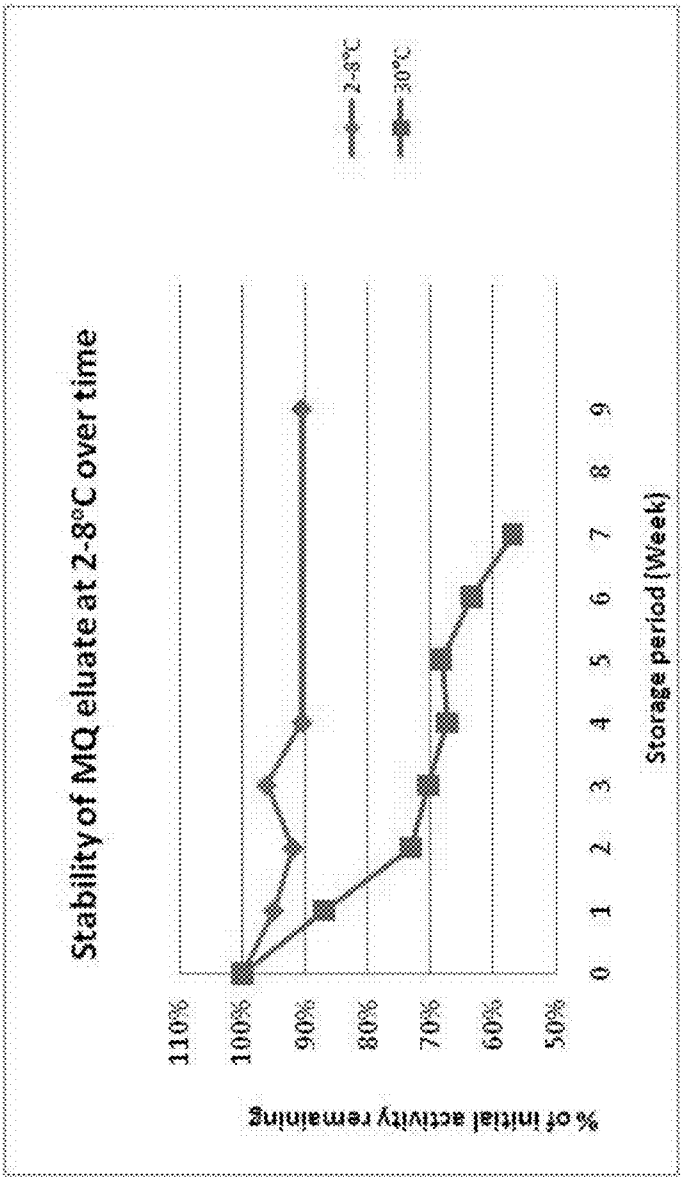
FIGURE 6



Processed Channel: 280NM

	Processed Channel	Retention Time (min)	Area	% Area	Height
1	280NM	14.798	1035217	3.42	12701
2	280NM	17.369	29350277	96.58	367205

FIGURE 7


Stability of MQ eluate at 2-8°C over time
2-8°C
30°C
% of initial activity remaining
110%
100%
90%
80%
70%
60%
50%
0 1 2 3 4 5 6 7 8 9
Storage period (Week)

METHOD OF PURIFYING THERAPEUTIC PROTEINS

CROSS REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/733,761, filed Dec. 5, 2012, which is hereby incorporated by reference.

TECHNICAL FIELD

[0002] The present invention relates generally to a method of reducing the level of impurities in a solution containing at least one therapeutic protein and to the resultant therapeutic-protein containing solutions. More specifically, to a method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a feedstock comprising fibrinogen and/or Factor VIII and/or Von Willebrand factor (VWF). The present invention also relates generally to solutions and pharmaceutical formulations comprising the fibrinogen and/or Factor VIII and/or VWF recovered by such methods, and uses thereof.

BACKGROUND

[0003] Current methods of purifying a naturally-occurring or recombinant therapeutic protein from a solution comprising said protein usually carry at least some impurities into the final preparation. In some instances, the presence of impurities, such as proteases, will destabilise the therapeutic protein in solution, particularly during storage. For this reason, many therapeutic proteins are stored as lyophilized or frozen preparations.

[0004] Whilst destabilising levels of impurities can affect many different types of therapeutic proteins, they are particularly relevant to those used for maintaining haemostasis. Haemostasis is an important physiological process that prevents bleeding following damage (e.g. a rupture) to blood vessels. There are three basic mechanisms that promote haemostasis: (i) vasoconstriction, (ii) platelet aggregation at the rupture site; and (iii) coagulation. During coagulation, damaged endothelial cells release tissue factor (Factor III), which in turn activates Factor VII with the aid of Ca^{2+}. Factor XII, which is released by activated platelets, activates Factor XI. Activated Factor VII and Factor XI promote a cascade of enzymatic reactions that lead to the activation of Factor X. Active Factor X (Factor Xa), along with Factor III, Factor V, Ca^{2+}, and platelet thromboplastic factor (PF_3), activate prothrombin activator. Prothrombin activator converts prothrombin to thrombin, which converts fibrinogen (Factor I) to fibrin, which forms an initial mesh over the site of damage. The initial mesh is then converted to a dense fibrin clot by Factor XIII, sealing the rupture until the site is repaired. During the coagulation cascade, thrombin will also activate Factor VIII, a glycoprotein pro-cofactor that in the circulation is mainly complexed to von Willebrand factor (VWF). Factor VIII interacts with Factor IXa to activate Factor X in the presence of Ca^{+2} and phospholipids.

[0005] A deficiency in the level of any one or more of the proteins involved in coagulation, including fibrinogen, Factor VIII, and/or von Willebrand factor (VWF) whether congenital or acquired, can lead to insufficient clotting of blood and the risk of haemorrhage. Current treatment options are limited to the administration of a pharmaceutical preparation of one or more therapeutic proteins, with a view to restoring endogenous levels of said proteins and maintaining haemostasis. However, existing pharmaceutical preparations, which are typically derived from donated blood plasma or a recombinant source, comprise zymogens and proteases (e.g., prothrombin, plasminogen, tissue plasminogen activator (tPA) and/or other proteases), which can destabilise the therapeutic proteins, such as fibrinogen, Factor VIII, or VWF during storage. As a consequence, such preparations are relatively unstable in aqueous solution, with long-term storage limited to lyophilized or frozen preparations.

[0006] For clinical applications, fibrinogen is typically purified from human plasma, where it accounts for only about 2-5% (1.5-4.0 g/L) of total plasma proteins. Traditionally, the purification of fibrinogen from plasma is carried out by classical plasma fractionation, where fibrinogen is cryo-precipitated from plasma followed by precipitation with either ethanol, ammonium sulphate, β alanine/glycine, polymers (e.g., polyethelene glycol) or low ionic strength solutions. Such methods can achieve relative high yield and homogeneity. Where a greater level of purity is required, chromatographic techniques are often employed. However, existing precipitation and chromatographic techniques amenable to commercial scale manufacturing processes typically produce fibrinogen preparations that comprise contaminating proteins such as zymogens or proteases (e.g., prothrombin, tissue plasminogen activator (tPA) and plasminogen), which can destabilize fibrinogen in solution. For example, when prothrombin is present, it can be activated to the serine protease thrombin which will in turn convert fibrinogen into fibrin. Similarly, when both tPA and plasminogen are present, tPA can activate plasminogen to its active form plasmin, which will in turn hydrolyse fibrinogen into fibrin. As a consequence, fibrinogen preparations are relatively unstable in aqueous solution, with long-term storage limited to lyophilized or frozen preparations.

[0007] Specific contaminants can be absorbed out; for example, fibronectin on immobilised gelatine and plasminogen on immobilised lysine (Vuento et al. 1979, Biochem. J., 183(2):331-337). However, the use of specific affinity resins is not amenable to large scale commercial processes. Reasons for this include the affinity resins themselves not being sufficiently robust to be used repeatedly and generally add significantly to the both processing time and costs.

[0008] EP1240200 (U.S. Pat. No. 6,960,463) is directed to methods of purifying fibrinogen from a fibrinogen-containing solution using ion exchange (IEX) chromatography. In particular the method involves applying a fibrinogen-containing solution to an ion exchange matrix under conditions that allow the fibrinogen to bind to the matrix and then washing the ion exchange matrix with a solution comprising at least one omega amino acid. This is done to promote the differential removal of plasminogen from the resin. Fibrinogen that is bound to the matrix is then eluted from the matrix.

[0009] WO2012038410 provides a method of purifying fibrinogen using anion exchange resins which contain a hydroxylated polymer support grafted with tertiary or quaternary amines that bind fibrinogen.

[0010] EP1519944 teaches the use of an immobilized metal ion affinity chromatography matrix under conditions that fibrinogen and plasminogen bind to the matrix, and selectively eluting the fibrinogen and plasminogen separately, such that the main fibrinogen fraction contains about 600 ng of plasminogen per mg of protein.

[0011] The present invention provides a method of reducing the level of plasminogen and/or tissue plasminogen acti-

vator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or VWF. The purified protein(s) are stable during storage as liquid preparations and can be used for clinical or veterinary applications, including treating or preventing conditions associated with a deficiency in the level of said protein(s).

SUMMARY OF THE INVENTION

[0012] In an aspect of the present invention, there is provided a method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or von Willebrand factor (VWF), the method comprising:

[0013] (i) passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a hydrophobic charge-induction chromatographic resin under conditions selected such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the resin; and

[0014] (ii) recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the resin;

[0015] wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or proteases(s) in the recovered solution is reduced by at least 50% compared to the feedstock.

[0016] In another aspect of the present invention, there is provided a method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or von Willebrand factor (VWF), the method comprising:

[0017] (i) passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a first hydrophobic charge-induction chromatographic resin;

[0018] (ii) recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the first hydrophobic charge-induction chromatographic resin;

[0019] (iii) passing the solution that is recovered in step (ii) through a second hydrophobic charge-induction chromatographic resin; and

[0020] (iv) recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the second hydrophobic charge-induction chromatographic resin;

[0021] wherein the conditions of the chromatographic steps are such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the first and/or second resin, and wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution that is recovered in step (iv) is reduced by at least 50% compared to the feedstock.

[0022] In another aspect, there is provided a solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the method of the present invention, as herein described.

[0023] In another aspect, there is provided a vessel containing at least 5 mL of a stable pharmaceutically acceptable fibrinogen solution, wherein the concentration of fibrinogen is at least 20 mg/mL.

[0024] In another aspect, there is provided a pharmaceutical formulation comprising a solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the method of the present invention, as herein described, and a pharmaceutically acceptable carrier.

[0025] In another aspect of the present invention, there is provided a solution comprising:

[0026] (a) at least 75% total protein of fibrinogen;

[0027] (b) less than 50 pg/mg total protein of tissue plasminogen activator; and

[0028] (c) less than 1 μg/mg total protein of plasminogen.

[0029] In another aspect of the present invention, there is provided a solution comprising:

[0030] (a) at least 90% total protein of fibrinogen;

[0031] (b) less than 50 pg/mg total protein of tissue plasminogen activator; and

[0032] (c) less than 150 ng/mg total protein of plasminogen.

[0033] In another aspect of the present invention, there is provided a solution comprising:

[0034] (a) at least 90% total protein of fibrinogen;

[0035] (b) less than 20 pg/mg total protein of tissue plasminogen activator; and

[0036] (c) less than 10 ng/mg total protein of plasminogen.

[0037] In another aspect, there is provided a method of treating or preventing a condition associated with fibrinogen deficiency, the method comprising administering to a subject in need thereof the solution or pharmaceutical formulation of the present invention, as herein described.

[0038] In another aspect, there is provided use of the solution of the present invention, as herein described, in the manufacture of a medicament for treating or preventing a condition associated with fibrinogen deficiency.

[0039] In another aspect, there is provided a fibrin glue comprising the solution of the present invention, as herein described.

[0040] In another aspect, there is provided a method of producing a stable liquid fibrinogen solution, the method comprising:

[0041] (i) passing a feedstock comprising fibrinogen through a hydrophobic charge-induction chromatographic resin under conditions selected such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the resin; and

[0042] (ii) recovering the solution comprising fibrinogen which passes through the resin;

wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or proteases(s) in the recovered solution is reduced by at least 50% compared to the feedstock.

[0043] In another aspect, there is provided a method of producing a stable liquid fibrinogen solution, the method comprising:

[0044] (i) passing a feedstock comprising fibrinogen through a first hydrophobic charge-induction chromatographic resin;

[0045] (ii) recovering the solution comprising fibrinogen which passes through the first hydrophobic charge-induction chromatographic resin;

[0046] (iii) passing the solution that is recovered in step (ii) through a second hydrophobic charge-induction chromatographic resin; and

[0047] (iv) recovering the solution comprising fibrinogen which passes through the second hydrophobic charge-induction chromatographic resin;

wherein the conditions of the chromatographic steps are such that the plasminogen and/or tissue plasminogen activator and/

or other protease(s) is bound to the first and/or second resin, and wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution that is recovered in step (iv) is reduced by at least 50% compared to the feedstock.

BRIEF DESCRIPTION OF THE FIGURES

[0048] This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0049] FIG. **1** shows the percentage recovery of fibrinogen, plasminogen, t-PA and Factor II from a fibrinogen solution over a range of pH levels when the solution is passed through a HEA Hypercel in negative mode with respect to fibrinogen.

[0050] FIG. **2** shows the percentage recovery of fibrinogen, plasminogen, t-PA, and Factor II from a fibrinogen solution over a range of pH levels when the solution is passed through a PPA Hypercel in negative mode with respect to fibrinogen.

[0051] FIG. **3** shows the percentage recovery of fibrinogen, plasminogen, t-PA, and Factor II from a fibrinogen solution over a range of pH levels when the solution is passed through a MEP Hypercel in negative mode with respect to fibrinogen.

[0052] FIG. **4***a* shows the percentage recovery of fibrinogen, plasminogen, t-PA and Factor II from a fibrinogen-containing solution over a range of pH levels when the solution is passed through a HEA Hypercel in negative mode with respect to fibrinogen.

[0053] FIG. **4***b* shows the stability of the fibrinogen containing solution recovered from the drop through fraction of the HEA Hypercel column (FIG. **4***a*) over 6 days at room temperature (approx. 20° C.), as measured by % of initial clottable protein.

[0054] FIG. **4***c* shows the stability of the fibrinogen containing solution recovered from the drop through fraction of the HEA Hypercel column (FIG. **4***a*) over 6 days at room temperature (approx. 20° C.), as measured by % clottable protein.

[0055] FIG. **5** shows the percentage process recovery for fibrinogen, t-PA, plasminogen and Factor II in fractions derived from a method according to an embodiment of the present invention, from the plasma cryoprecipitate through to the Macroprep-HQ eluate.

[0056] FIG. **6** shows the purity of monomeric fibrinogen that was recovered from the Macroprep-HQ chromatographic resin, as measured by analytical size exclusion HPLC chromatogram.

[0057] FIG. **7** shows the fibrinogen activity as a percentage of the initial fibrinogen activity at t=0 of liquid fibrinogen recovered from the Macroprep-HQ chromatographic resin that is stored at either 2-8° C. for a 4 week period or 30° C. for a 5 week period.

DETAILED DESCRIPTION

[0058] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0059] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

[0060] It must be noted that, as used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a formulation" includes a single formulation, as well as two or more formulations.

[0061] In the absence of any indication to the contrary, reference made to a "%" content throughout this specification is to be taken as meaning % w/w (weight/weight). For example, a solution comprising at least 80% total protein of fibrinogen is taken to mean a solution comprising fibrinogen at a concentration of at least 80% w/w of total protein. This can be calculated for example by dividing the amount of fibrinogen derived from the clottable protein assay by the total protein amount derived from a standard protein assay (e.g. Biuret) and multiplying by 100. In the clottable protein assay, thrombin is added to a sample to form a clot, which is almost all fibrin. The clot can be separated from the supernatant containing non-clottable proteins by centrifugation. Subsequently the clot is washed and dissolved by alkaline urea or other substances and the protein concentration is determined by spectrophotometry. Since the majority of the clot is fibrin, the protein concentration will be equivalent to the fibrinogen concentration. Hence the amount of clottable protein in a sample is equivalent to the difference between the total protein and the non-clottable protein component of the sample.

[0062] The purification of fibrinogen and/or Factor VIII and/or VWF from a feedstock (e.g., plasma or cell culture supernatant) is typically carried out by conventional fractionation, where the fibrinogen and/or Factor VIII and/or VWF is precipitated from the solution using, for example, ethanol, ammonium sulphate, 13 alanine/glycine, polymers (e.g., polyethylene glycol) and/or low ionic strength solutions. Current plasma purification methods employing different chromatographic steps can achieve relative high yield and homogeneous preparations of a protein of interest. However, these methods typically result in preparations comprising residual amounts of impurities that may not be suitable for formulating a stable liquid preparation for clinical applications Impurities such as prothrombin, tissue plasminogen activator (tPA) and plasminogen are particularly problematic, as destabilizing levels of these impurities can hydrolyse fibrinogen in aqueous solution, thus rendering the fibrinogen unstable, particularly during manufacture and/or long-term storage.

[0063] The present invention is predicated, at least in part, on the finding that passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a hydrophobic charge-induction chromatographic (HCIC) resin and recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF that passes through the resin is an efficient alternative to existing purification processes for reducing the destabilizing level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution.

[0064] Thus, in an aspect of the present invention, there is provided a method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or von Willebrand factor (VWF), the method comprising:

[0065] (i) passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a hydrophobic

charge-induction chromatographic resin under conditions selected such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the resin; and

[0066] (ii) recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the resin;

wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or proteases(s) in the recovered solution is reduced by at least 50% compared to the feedstock.

[0067] In an embodiment, the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the recovered solution comprising fibrinogen and/or Factor VIII and/or VWF is reduced by at least 60%, by at least 70%, by at least 80%, or by at least 90% or by at least 95% or by at least 98% compared to the feedstock.

[0068] In another aspect, there is provided a method of producing a stable liquid fibrinogen solution, the method comprising:

[0069] (i) passing a feedstock comprising fibrinogen through a hydrophobic charge-induction chromatographic resin under conditions selected such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the resin; and

[0070] (ii) recovering the solution comprising fibrinogen which passes through the resin;

wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or proteases(s) in the recovered solution is reduced by at least 50% compared to the feedstock.

[0071] In an embodiment, the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the recovered solution comprising fibrinogen is reduced by at least 60%, by at least 70%, by at least 80%, or by at least 90% or by at least 95% or by at least 98% compared to the feedstock.

[0072] Chromatographic processes typically employ a solid support, also referred to interchangeably herein as a resin or matrix. Suitable solid supports would be familiar to persons skilled in the art. Examples include inorganic carriers, such as glass and silica gel, organic, synthetic or naturally occurring carriers, such as agarose, cellulose, dextran, polyamide, polyacrylamides, vinyl copolymers of bifunctional acrylates, and various hydroxylated monomers, and the like. Commercially available carriers are sold under the names of Sephadex™, Sepharose™, Hypercel™, Capto™, Fractogel™, MacroPrep™, Unosphere™, GigaCap™, Trisacryl™, Ultrogel™, Dynospheres™, Macrosorb™ and XAD™ resins.

[0073] The chromatography steps will generally be carried out under non-denaturing conditions and at convenient temperatures in the range of about +10° C. to +30° C., more usually at about ambient temperatures. The chromatographic steps may be performed batch-wise or continuously, as convenient. Any convenient method of separation may be employed, such as column, centrifugation, filtration, decanting, or the like.

[0074] Hydrophobic Charge Induction Chromatography (HCIC) which is often also referred to as either mixed mode, or multimodal chromatography, will be familiar to persons skilled in the art. HCIC uses binding moieties attached to a solid support, wherein the binding moieties may have specificity for one or more proteins that, in accordance with the methods of the present invention, represent impurities in the feedstock (e.g., zymogens and proteases such prothrombin, tPA and plasminogen).

[0075] Any suitable HCIC resin known to persons skilled in the art can be used. In an embodiment, the HCIC resin comprises a ligand selected from the group consisting of mercaptoethylpyridine (4-mercaptoethylpyridine, e.g., MEP Hypercel), n-hexylamine (e.g., HEA Hypercel) and phenylpropylamine (e.g., PPA Hypercel). In an embodiment, the HCIC resin comprises n-hexylamine.

[0076] HCIC ligands such as HEA, MEP and PPA have an advantage in that they permit separation based on the surface hydrophobicity of proteins, but do not require the addition of lyotropic salts often seen in other processes for the purification of fibrinogen using hydrophobic chromatography (e.g., hydrophobic interaction chromatography; HIC). In contrast to traditional hydrophobic interaction chromatography, HCIC is controlled on the basis of pH, rather than salt concentration. HCIC resins also provide high binding capacity and high flow rates, ideal for both laboratory- and industrial-scale purification.

[0077] HCIC resins are often packed into columns with bed heights from about 2 cm to about 40 cm. At industrial scale the bed heights are usually at least 10 cm and are typically in the range from about 15 cm to 25 cm. The column diameters of industrial columns can range from 20 cm up to about 1.5 m. Such columns are operated at flow rates in accordance with HCIC resin manufacture instructions with flow rates in the 50-100 cm/hr range typical. The upper flow rate limitation is in part due to HCIC resin pressure constraints. For HEA resin for example the upper operating pressure limit is <3 bar (<300 kPa). Typical dynamic binding capacities (10% breakthrough of a binding protein) for HCIC resins are about 20 to 30 mg of bound protein per mL of resin. For the present invention, this enables relative large amounts of protein to be loaded onto the HCIC column as the abundant proteins like fibrinogen can pass through the column whilst less abundant proteins such as plasminogen and/or tissue plasminogen activator and/or other protease(s) bind to the HCIC resin, This is advantageous for industrial scale manufacture as either smaller sized columns and/or less column cycles are required to process a batch.

[0078] The present inventors have also found that the pH of the solution or feedstock comprising fibrinogen and/or Factor VIII and/or VWF that is passed through the HCIC resin in accordance with the methods of the present invention can be adjusted to control the recovery of the fibrinogen and/or Factor VIII and/or VWF and removal of impurities. Thus, in an embodiment disclosed herein, the solution or feedstock comprising fibrinogen and/or Factor VIII and/or VWF that is passed through the HCIC resin has a pH from about 6.0 to about 9.5. In particular embodiments the solution or feedstock comprising fibrinogen and/or Factor VIII and/or VWF is passed through the HCIC resin preferably at a pH of about 6.0, 6.25, 6.5, 6.75, 7.0, 7.25, 7.5, 7.75, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, or 9.5. In a particular embodiment, the solution or feedstock comprising fibrinogen and/or Factor VIII and/or VWF that is passed through the HCIC resin has a pH of about 7.0. In particular embodiments, the HCIC resin is equilibrated prior to loading of the solution or feedstock at a pH of about 6.0, 6.25, 6.5, 6.75, 7.0, 7.25, 7.5, 7.75, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, or 9.5.

[0079] The method of the present invention may also employ the use of more than one additional chromatography

step to remove further impurities, if necessary, and thus improve the purity of the final preparation. Additional chromatographic purification steps can be implemented either before or after the purification of fibrinogen and/or Factor VIII and/or VWF through the HCIC resin in accordance with the present invention. For example, the solution comprising fibrinogen and/or Factor VIII and/or VWF that is recovered from the HCIC resin in step (ii) can be passed through another chromatographic resin.

[0080] The additional chromatographic purification steps may employ another HCIC resin. Thus, in another aspect of the present invention, there is provided a method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or VWF, the method comprising:

[0081] (i) passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a first hydrophobic charge-induction chromatographic resin;

[0082] (ii) recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the first hydrophobic charge-induction chromatographic resin;

[0083] (iii) passing the solution that is recovered in step (ii) through a second hydrophobic charge-induction chromatographic resin; and

[0084] (iv) recovering the solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the second hydrophobic charge-induction chromatographic resin;

wherein the conditions of the chromatographic steps are such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the first and/or second resin, and wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution that is recovered in step (iv) is reduced by at least 50% compared to the feedstock.

[0085] In an embodiment, the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution comprising fibrinogen and/or Factor VIII and/or VWF that is recovered in step (iv) is reduced by at least 60%, by at least 70%, by at least 80%, or by at least 90% or by at least 95% or by at least 98% compared to the feedstock.

[0086] In another aspect, there is provided a method of producing a stable liquid fibrinogen solution, the method comprising:

[0087] (i) passing a feedstock comprising fibrinogen through a first hydrophobic charge-induction chromatographic resin;

[0088] (ii) recovering the solution comprising fibrinogen which passes through the first hydrophobic charge-induction chromatographic resin;

[0089] (iii) passing the solution that is recovered in step (ii) through a second hydrophobic charge-induction chromatographic resin; and

[0090] (iv) recovering the solution comprising fibrinogen which passes through the second hydrophobic charge-induction chromatographic resin;

wherein the conditions of the chromatographic steps are such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the first and/or second resin, and wherein the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution that is recovered in step (iv) is reduced by at least 50% compared to the feedstock.

[0091] In an embodiment, the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution comprising fibrinogen that is recovered in step (iv) is reduced by at least 60%, by at least 70%, by at least 80%, or by at least 90% or by at least 95% or by at least 98% compared to the feedstock.

[0092] In an embodiment disclosed herein, the second HCIC resin is different from the first HCIC resin. In another embodiment disclosed herein, the first and second hydrophobic charge-induction chromatographic resins are the same. Where the solution comprising fibrinogen and/or Factor VIII and/or VWF that is recovered from the HCIC resin in step (ii) is passed through the same HCIC resin, it may be desirable to wash the HCIC resin after step (ii) and prior to passing the recovered solution through the HCIC resin in step (iii) again in order to remove any impurities that may be bound to the resin.

[0093] The additional chromatographic resin may also be an anion exchange chromatographic resin. In anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. A positively charged solid support can be prepared by any means known to persons skilled in the art and will usually involve the covalent attachment of a negatively charged functional ligand onto a solid support. Suitable negatively charged functional ligands will invariably depend on the molecule to be separated from solution. Examples of suitable anion exchange resins are ones comprising a functional quaternary amine group (Q) and/or a tertiary amine group (DEAE), or a diethylaminopropyl group (ANX). In an embodiment disclosed herein, the anion exchange resin is a strong anion exchange resin. In another embodiment disclosed herein, the strong anion exchange resin comprises a quaternary amine functional ligand (e.g., —N+(CH3)3 as seen, for example, in Macroprep-HQ™; Bio-Rad Laboratories). In yet another embodiment the anion exchange resin is trimethylamine groups grafted to a hydroxylated methacrylic polymer via a linking group such as GigaCap Q-650M®.

[0094] In an embodiment, anion exchange chromatography is performed in positive mode with respect to the fibrinogen and/or Factor VIII and/or VWF. That is, the conditions used are such that, when the solution or feedstock comprising fibrinogen and/or Factor VIII and/or VWF is passed through the anion exchange chromatographic resin, the fibrinogen and/or Factor VIII and/or VWF bind(s) to the positively-charged functional groups attached to the resin, allowing impurities in the solution to pass through the resin in the flow-through (drop-through) fraction, where they can be discarded or recovered for other purposes. Once the flow-through fraction passes through the resin, the anion exchange chromatographic resin can be washed with a suitable wash buffer known to persons skilled in the art. The constituents of the wash buffer and the conditions of the wash step will typically be selected to retain the fibrinogen and/or Factor VIII and/or VWF bound to the resin during the wash step.

[0095] In an embodiment disclosed herein, prior to eluting the fibrinogen and/or Factor VIII and/or VWF from the anion exchange chromatographic resin, the resin is washed with a wash solution comprising epsilon-aminocaproic acid (ϵ-ACA). The addition of ϵ-ACA to the wash buffer can promote the elution of proteases (such as plasminogen) that may be bound to the anion exchange chromatographic resin

during the first pass. An example of a suitable wash step is described in U.S. Pat. No. 6,960,463.

[0096] For eluting the fibrinogen and/or Factor VIII and/or VWF that remains bound to the anion exchange chromatographic resin, any suitable elution buffer known to persons skilled in the art can be used. For the removal of plasminogen and/or t-PA and/or other protease(s) from a solution comprising fibrinogen, the present inventors have found that an elution buffer comprising from about 150 mM to about 300 mM NaCl allows fibrinogen monomers to be eluted from the anion exchange resin while minimizing the elution of fibrinogen aggregates and/or other proteins (e.g., Factor VIII, VWF, fibronectin or proteases) that may be also bound to the resin. Thus, in an embodiment disclosed herein, the fibrinogen is eluted from the anion exchange resin with an elution buffer comprising from about 150 mM to about 300 mM NaCl. This equates to an elution buffer having a conductivity range of about 14 mS/cm (150 mM NaCl) to about 25 mS/cm (300 mM NaCl).

[0097] In another embodiment, the fibrinogen is eluted from the anion exchange resin with an elution buffer comprising from about 150 mM to about 270 mM NaCl. This equates to an elution buffer having a conductivity range of about 14 mS/cm (150 mM NaCl) to about 23 mS/cm (270 mM NaCl).

[0098] In another embodiment, the fibrinogen is eluted from the anion exchange resin with an elution buffer comprising from about 170 mM to about 230 mM NaCl. This equates to an elution buffer having a conductivity range of about 15 mS/cm (170 mM NaCl) to about 20 mS/cm (230 mM NaCl).

[0099] In another embodiment, the fibrinogen is eluted from the anion exchange resin with an elution buffer comprising from about 200 mM to about 220 mM NaCl. This equates to an elution buffer having a conductivity range of about 18 mS/cm (200 mM NaCl) to about 19 mS/cm (220 mM NaCl).

[0100] In another embodiment, the fibrinogen is eluted from the anion exchange resin with an elution buffer comprising from about 150 mM to about 190 mM NaCl.

[0101] In an embodiment disclosed herein, the elution buffer comprises a free amino acid at a concentration that promotes the elution of fibrinogen monomer over aggregates thereof. In another embodiment, the elution buffer comprises a free amino acid at a concentration of about 1 to 3% (w/v). Any suitable free amino acid may be used in this capacity. In an embodiment, the free amino acid is arginine.

[0102] In an embodiment, anion exchange chromatography is performed in negative mode with respect to the fibrinogen and positive mode in respect to Factor VIII and/or VWF. That is, the conditions used are such that, when the solution or feedstock comprising fibrinogen and Factor VIII and/or VWF is passed through the anion exchange chromatographic resin, the Factor VIII and/or VWF bind(s) to the positively-charged functional groups attached to the resin, allowing fibrinogen in the solution to pass through the resin in the flow-through (drop-through) fraction. Once the fibrinogen containing flow-through fraction passes through the resin, the anion exchange chromatographic resin can be washed with a suitable wash buffer known to persons skilled in the art. The constituents of the wash buffer and the conditions of the wash step will typically be selected to retain the Factor VIII and/or VWF bound to the resin during the wash step.

[0103] In an embodiment, the solution or feedstock comprising fibrinogen and/or Factor VIII and/or VWF is passed through the anion exchange chromatographic resin in the presence of about 150 mM to about 270 mM NaCl. This equates to a conductivity range of about 14 mS/cm (150 mM NaCl) to about 23 mS/cm (270 mM NaCl). Under these conditions the fibrinogen particularly the monomeric form, passes through the anion exchange chromatographic resin whilst fibrinogen containing aggregates and other impurities such as IgG and fibronectin bind to the resin. In further embodiments the solution or feedstock comprising fibrinogen and/or Factor VIII and/or VWF is passed through the anion exchange chromatographic resin in the presence of about 170 mM to about 230 mM NaCl (about 15 mS/cm to about 20 mS/cm) or about 200 mM to about 220 mM NaCl (about 18 mS/cm to about 19 mS/cm). Under these types of conditions it is expected that Factor VIII and/or vWF will bind to the anion exchange chromatographic resin.

[0104] Factor VIII and/or VWF can be eluted from the anion exchange resin with an elution buffer comprising at least 300 mM of a salt such as NaCl. In a particular embodiment Factor VIII and/or VWF are eluted from the anion exchange resin with about 500 mM NaCl. Where fibrinogen and Factor VIII and/or VWF are bound to the anion exchange resin, the elution step can be conducted such that the fibrinogen is initially eluted (for example using conditions set out in the embodiments above) and then the Factor VIII and/or VWF can be eluted using a higher concentration of salt such as 500 mM NaCl.

[0105] Where an anion exchange chromatography step is employed, it can be performed either before and/or after passing the feedstock comprising fibrinogen and/or Factor VIII and/or VWF through the HCIC resin. In an embodiment disclosed herein, the method further comprises passing the solution comprising fibrinogen and/or Factor VIII and/or VWF that is recovered in step (ii) through an anion exchange chromatographic resin. In another embodiment, where first and second HCIC chromatographic steps are employed, as herein described, the method further comprising passing the solution comprising fibrinogen and/or Factor VIII and/or VWF that is recovered in step (ii) and/or step (iv) through an anion exchange chromatographic resin.

[0106] In an embodiment disclosed herein, the method further comprises passing the feedstock comprising fibrinogen and/or Factor VIII and/or VWF through an anion exchange chromatographic resin prior to step (i).

[0107] Persons skilled in the art will understand that the number of additional chromatographic steps used in accordance with the present invention will depend on the level of purity required in the final preparation. For example, the method of the present invention may comprise 2, 3, 4 or 5 chromatography steps, as disclosed herein. For example, where the method comprises 2 chromatography steps, the sequence of steps will be HCIC/IEX or HCIC/HCIC or IEX/HCIC; where the method comprises 3 chromatography steps, the sequence of steps will be HCIC/IEX/HCIC or HCIC/HCIC/IEX or HCIC/HCIC/HCIC or HCIC/IEX/IEX or IEX/HCIC/HCIC or IEX/HCIC/IEX or IEX/IEX/HCIC; where the method comprises 4 chromatography steps, the sequence of steps will be HCIC/IEX/HCIC/HCIC or HCIC/HCIC/IEX/HCIC or HCIC/HCIC/HCIC/IEX or HCIC/HCIC/HCIC/HCIC or HCIC/IEX/IEX/HCIC or HCIC/IEX/IEX/IEX or HCIC/HCIC/IEX/IEX or HCIC/IEX/HCIC/IEX or IEX/HCIC/IEX/HCIC or IEX/HCIC/HCIC/IEX or IEX/

HCIC/HCIC/HCIC or IEX/HCIC/IEX/IEX or IEX/IEX/HCIC/HCIC or IEX/IEX/HCIC/IEX or IEX/IEX/IEX/HCIC; and so forth (where "IEX" denotes anion exchange chromatography). The required level of purity may be dictated by the intended use of the solution (e.g., for treatment of patient with a fibrinogen and/or Factor VIII and/or VWF deficiency) and/or where a longer storage period is required as an aqueous preparation.

[0108] Chromatography can be performed using any means known to persons skilled in the art. For example, the chromatography steps according to the present invention can use axial flow columns, such as those available from GE Healthcare, Pall Corporation and Bio-Rad, or radial flow columns, such as those available from Proxcys. The chromatography steps according to the present invention can also be conducted using expanded bed technologies.

[0109] In an embodiment, the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the recovered solution comprising fibrinogen and/or Factor VIII and/or VWF is reduced by at least 60%, by at least 70%, by at least 80%, or by at least 90% or by at least 95% compared to the feedstock.

[0110] Methods that maximize the removal of impurities such as plasminogen and/or tissue plasminogen activator and/or other protease(s) are particularly advantageous, because the stability and efficacy of the fibrinogen and/or Factor VIII and/or VWF in solution is decisively improved, particularly during long-term storage. Storage in liquid form is particularly advantageous for solutions comprising fibrinogen and/or Factor VIII and/or VWF because immediate use in a patient is possible. This is in contrast to the use of lyophilised preparations of purified fibrinogen and/or Factor VIII and/or VWF, which require reconstituting the lyophilized protein(s) in a suitable buffer and/or water for injection immediately prior to administration into a subject in need thereof.

[0111] An advantage of depleting proteases or their zymogens (such as plasminogen) from a solution comprising fibrinogen and/or Factor VIII and/or VWF is that it minimises the need to add anti-fibrinolytic agents to inhibit any residual protease and/or zymogen (e.g., plasmin or plasminogen). Examples of such agents include aprotinin, a bovine protein inhibitor of plasmin; ortranexamic acid, a synthetic plasmin inhibitor also associated with neurotoxic side-effects.

[0112] A further advantageous feature is that plasminogen, which has been separated from the solution comprising fibrinogen and/or Factor VIII and/or VWF by HCIC, may be further processed to yield a plasminogen-containing concentrate for, for example, clinical use. HCIC may therefore be used to prepare both plasminogen and solutions comprising fibrinogen and/or Factor VIII and/or VWF from a single starting solution.

[0113] A further advantageous feature is that the production costs of the HCIC resin is far more economical than the cost of lysine-Sepharose or immobilised lysine resin which are used in affinity chromatography procedures.

[0114] A further advantageous feature is that the HCIC could be used to replace aluminium hydroxide (e.g. Alhydrogel) steps for the removal of proteases (e.g Factor II). Alhydrogel is currently widely used in the commercial production of Factor VIII and VWF. The material is, however, relatively costly with 100 kg's typically used per batch. Moreover, alhydrogel often requires manual handling and the material is discarded after a single use. In contrast, HCIC steps can be fully automated and the resin can be used in the manufacture of multiple batches.

[0115] Another advantageous feature is that the HCIC resin is compatible with 1M NaOH which can be used for inactivation and removal of pathogens including viruses and prions during column cleaning and resin sanitisation procedures.

[0116] Liquid preparations derived from the methods of the present invention also have advantages over the use of frozen preparations, which require expensive storage and transport means and must be thawed prior to immediate use. Even where the fibrinogen and/or Factor VIII and/or VWF is stored as a lyophilized or frozen preparation, it is advantageous for the reconstituted or thawed protein to be stable for longer. This is evident, for example, where material has been reconstituted as a precaution for a medical procedure, but its use was not required on the basis of medical considerations. This material is typically discarded, as the fibrinogen is only stable over a short-term period due to the presence of prothrombin and/or t-PA and/or other protease(s).

[0117] The solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the methods of the present invention are advantageous because they provide a preparation of fibrinogen and/or Factor VIII and/or VWF that has greater stability than existing lyophilised preparations, even at room temperature. This can be particularly advantageous on lengthy transport routes where low temperatures may, where appropriate, not be ensured throughout transport and/or storage. Stable storage of fibrinogen and/or Factor VIII and/or VWF in solution also facilitates, in many respects, the production, usage, transport and administration to a patient in need thereof. Owing to the increased stability of the fibrinogen and/or Factor VIII and/or VWF prepared in accordance with the present invention, it is possible in many pharmaceutical preparations to dispense with the addition of stability agents, such as fibrinolysis or fibrinogenolysis inhibitors that may, in some circumstances, lead to unwanted side effects or which should be avoided to reduce potential risks.

[0118] The term "stable", as used herein, means that there is little or no substantial loss of activity of the fibrinogen and/or Factor VIII and/or VWF after a period of time in storage as compared to the level of activity of the fibrinogen and/or Factor VIII and/or VWF before storage (e.g., as compared to the level of activity determined immediately after recovery of the solution comprising fibrinogen and/or Factor VIII and/or VWF in accordance with the present invention). In an embodiment disclosed herein, the solution comprising fibrinogen and/or Factor VIII and/or VWF retains at least 70% activity, preferably at least 80% activity, more preferably at least 90% activity, even more preferably at least 95% activity and most preferably 100% activity after a period of time in storage at a temperature of about 0° C. to about 30° C.

[0119] In an embodiment disclosed herein, the fibrinogen recovered by the methods of the present invention retains from about 90% to 100% activity after at least 4 weeks in storage at a temperature of about 2° C. to about 8° C., preferably retains about 90% activity after 4 weeks in storage at a temperature of about 2° C. to about 8° C. In another embodiment disclosed herein, the fibrinogen retains from about 60% to about 80% activity after at least 4 weeks in storage at a temperature of about 30° C., preferably retains from about 60% to about 70% activity after 5 weeks in storage at a temperature of about 30° C.

[0120] The level of activity of the fibrinogen and/or Factor VIII and/or VWF can be determined by any means known to persons skilled in the art. Examples of suitable methods for determining the activity of fibrinogen, for example, are summarised by Mackie et al. (British J. Haematol. 121:396-404, 2003). Particular methods include Clauss (Clauss, 1957, Acta-Haematol. 17, 237-246) and/or clottable protein (Jacobsson K., Scand J Clin Lab Invest 1955; 7 (supp 14):1-54 or Fibrin sealant Ph. Eur. Monograph 903, 2012). Results can be reported as % clottable protein; % of initial clottable protein, and/or % of initial fibrinogen activity as determined using the Clauss method or similar.

[0121] The skilled person will understand that the concentration of the plasminogen and/or tissue plasminogen activa-

tor and/or other protease(s) in the recovered solution comprising fibrinogen and/or Factor VIII and/or VWF is likely to dictate the length of storage and/or storage conditions (e.g., temperature). For example, it will be understood that a preparation in which the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the recovered solution is reduced by 80% compared to the feedstock may be stored for a longer period of time and/or at higher temperatures without significantly destabilising the activity of the fibrinogen and/or Factor VIII and/or VWF as compared to a preparation in which the concentration of the plasminogen and/or tissue plasminogen activator and/or other protease(s) in the recovered solution comprising fibrinogen and/or Factor VIII and/or VWF is reduced by only 50% compared to the feedstock.

[0122] Whilst the methods of the present invention can be performed at laboratory scale, they can be scalable up to industrial size without significant changes to conditions. Thus, in an embodiment disclosed herein, the methods of the present invention are performed on an industrial or commercial scale. Preferably, the methods of the invention are suitable for the commercial scale manufacture of fibrinogen and/or Factor VIII and/or VWF. For example, when using plasma fractions as a starting material in the method of the invention, then commercial scale manufacture would involve the use of a plasma fraction derived from at least about 500 kg of plasma. More preferably, the starting plasma fraction will be derived from at least about 5,000 kg, 7,500 kg, 10,000 kg and/or 15,000 kg of plasma per batch. In particular embodiments, the solutions and pharmaceutical formulations comprising fibrinogen and/or Factor VIII and/or VWF of the present invention are manufactured at commercial scale from a plasma fraction or a recombinant feedstock.

[0123] Where a solution comprising fibrinogen and/or Factor VIII and/or VWF is to be used for clinical or veterinary applications (e.g., for administration to a subject with fibrinogen and/or Factor VIII and/or VWF deficiency or for use as a fibrin glue), persons skilled in the art will understand that it may be desirable to reduce the level of active virus content (virus titre) and other potential infectious agents (for example prions) in the solution. This may be particularly desirable where the feedstock comprising fibrinogen and/or Factor VIII and/or VWF (i.e., the starting material) is derived from blood plasma. Methods of reducing the virus titre in a solution will be known to persons skilled in the art. Examples include pasteurization (for example, incubating the solution at 60° C. for 10 hours in the presence of high concentrations of stabilisers such as glycine (e.g. 2.75M) and sucrose (e.g. 50%) and/or other selected excipients or salts), dry heat treatment, virus filtration (passing the solution through a nano-filter; e.g., 20 nm cutoff) and/or subjecting the solution to treatment with a suitable organic solvent and detergent for a period of time and under conditions to inactivate virus in the solution. Solvent detergent has been used for over 20 years to inactivate enveloped viruses particularly in plasma-derived products including fibrinogen and factor VIII and/or VWF. Thus it may be carried out using various reagents and methods known in the art (see, for example, U.S. Pat. No. 4,540,573 and U.S. Pat. No. 4,764,369 which are hereby incorporated by reference). Suitable solvents include tri-n-butyl phosphate (TnBP) and ether, preferably TnBP (typically at about 0.3%). Suitable detergents include polysorbate (Tween) 80, polysorbate (Tween) 20 and Triton X-100 (typically at about 0.3%). The selection of treatment conditions including solvent and detergent concentrations depend in part on the characteristics of the feedstock with less pure feedstocks generally requiring higher concentrations of reagents and more extreme reaction conditions. A preferred detergent is polysorbate 80 and a particularly preferred combination is polysorbate 80 and TnBP. The feedstock may be stirred with solvent and detergent reagents at a temperature and for a time sufficient to inactivate any enveloped viruses that may be present. For example, the solvent detergent treatment may be carried out for about 4 hours at 25° C. The solvent detergent chemicals are subsequently removed by for example adsorption on chromatographic media such as C-18 hydrophobic resins or eluting them in the drop-through fraction of ion exchange resins under conditions which adsorb the protein of interest.

[0124] The virus inactivation step can be performed at any suitable stage of the methods disclosed herein. In an embodiment, the feedstock comprising fibrinogen and/or Factor VIII and/or VWF is subject to a viral inactivation step prior to step (i). In another embodiment, the solution comprising fibrinogen and/or Factor VIII and/or VWF that is recovered from the hydrophobic charge-induction chromatographic resin (i.e., from steps (ii) and/or (iv)) is subject to a viral inactivation step. In an embodiment disclosed herein, the viral inactivation step comprises pasteurisation or treatment with an organic solvent and detergent. In another embodiment disclosed herein, the virus inactivation step comprises virus filtration. Where virus filtration is used, the inventors have found that the addition of a free amino acid (e.g., arginine) prior to the filtration step can significantly improve the flux rate and recovery of fibrinogen and/or Factor VIII and/or VWF through the filter. An example of such method is described in U.S. Pat. No. 7,919,592.

[0125] In an embodiment disclosed herein, the feedstock or solution comprising fibrinogen and/or Factor VIII and/or VWF is subject to a viral inactivation step before it is passed through the anion exchange chromatographic resin. The advantage of employing a virus inactivation step such as solvent detergent treatment prior to passing the treated solution or feedstock through an anion exchange chromatographic resin is that the anion exchange resin allows for the removal of the organic solvent and detergent from the treated solution by utilizing conditions that promote binding of the fibrinogen and/or Factor VIII and/or VWF to the resin and removal of the organic solvent and detergent with the flow-through (drop-through) fraction.

[0126] Pasteurization can generate protein aggregates and polymers, particularly in a solution comprising fibrinogen (also referred to herein as a "fibrinogen solution"). Therefore, it may be desirable in some instances to reduce the level of aggregates/polymers in a pasteurized solution. This can be achieved by any means knows to persons skilled in the art, although conveniently can be achieved by further chromatographic purification. In an embodiment disclosed herein, the pasteurized solution or feedstock is passed through an anion exchange chromatographic resin in positive mode with respect to the fibrinogen and/or Factor VIII and/or VWF such that any aggregates or polymers are removed with the flow-through (drop-through) fraction.

[0127] The term "feedstock" is used herein to denote any solution comprising fibrinogen and/or Factor VIII and/or VWF. The feedstock may also comprise other proteins (e.g., therapeutic proteins) known to persons skilled in the art. Examples include proteins involved in the blood coagulation cascade. In an embodiment disclosed herein, the feedstock comprises fibrinogen.

[0128] Suitable feedstock comprising fibrinogen and/or Factor VIII and/or VWF will be known to persons skilled in the art. Examples include plasma or plasma fractions such as solubilised plasma cryoprecipitate or solubilised Fraction I paste derived from human or animal plasma or a plasma fraction, cell culture fractions from recombinant technology, fractions derived from milk from transgenic animals, etc. Sources of recombinant fibrinogen and/or Factor VIII and/or VWF proteins are also suitable for use as a feedstock in accordance with the present invention. Where the feedstock is plasma or a plasma fraction, it can be either pooled plasma

donations or it can be from an individual donor. In an embodiment disclosed herein, the feedstock comprising fibrinogen and/or Factor VIII and/or VWF is a solubilised plasma cryoprecipitate. This component, either derived from whole blood or collected via apheresis, is prepared by controlled thawing fresh frozen plasma between 1-6° C. and recovering the precipitate. The cold-insoluble precipitate is refrozen. One unit of cryoprecipitate apheresis is approximately equivalent to 2 units of cryoprecipitate derived from whole blood. It contains most of the fibrinogen, Factor VIII and VWF along with other proteins such as factor XIII and fibronectin from fresh frozen plasma. An alternate source of fibrinogen is Fraction I precipitate which can be prepared from frozen plasma by thawing and removing the cryoprecipitate by either centrifugation or filtration. The resultant cryosupernatant is then mixed with ethanol to precipitate Fraction I. For example, a Fraction I precipitate can be obtained by adding about 8% (v/v) ethanol at pH 7.2 and controlling the temperature to about −3° C. (Cohn, et al. 1946, J. Am. Chem. Soc. 62: 459-475). In an embodiment, the feedstock comprising fibrinogen and/or Factor VIII and/or VWF is cryoprecipitate.

[0129] Reference in the specification to "protease(s)" can be to any protease and/or its zymogen present in the feedstock or solution comprising fibrinogen and/or Factor VIII and/or VWF that, when exposed to a HCIC resin, is capable of binding to HCIC resin under conditions where the fibrinogen and/or Factor VIII and/or VWF passes through the resin. Proteases may be any type, including serine proteases (e.g., plasmin, thrombin, trypsin), threonine proteases, cysteine proteases (e.g., cathepsin B and cathepsin H), aspartate proteases (e.g., pepsin), metallo-proteases (e.g., collagenases and gelatinases) and glutamic proteases. Where the feedstock comprising fibrinogen and/or Factor VIII and/or VWF is derived from human or animal plasma, the proteases/zymogens may include plasminogen, tissue plasminogen activator (tPA), thrombin, elastase, Factor VIIa, Factor IXa, Factor Xa, Factor XIa, Factor XIIa, Factor XIIIa, plasma kallikreins and the like. In regard to solutions comprising fibrinogen and/or Factor VIII and/or VWF, a particular preferred protease/zymogen to be removed is plasminogen. Other preferred proteases/zymogens to be removed from a solution comprising fibrinogen and/or Factor VIII and/or VWF are t-PA, pro- and/or active thrombin (Factor II/IIa). When the feedstock comprising fibrinogen and/or Factor VIII and/or VWF is derived from cell culture supernatant, the protease/zymogen can include any host cell protease, such as serine proteases (e.g., caseinases), metalloproteases (e.g., gelatinases, matrix metalloproteases (MMP) including MMP3, MMP10 or MMP12), aspartic proteases (cathepsin D), acid proteases amongst others.

[0130] In some instances, it may be desirable to remove or reduce the level of impurities from the feedstock before passing the feedstock through an HCIC resin in step (i). Removing or reducing the level of impurities from the feedstock can reduce the load on the HCIC resin during chromatographic purification and thus improve the efficiency of separation of plasminogen and/or tissue plasminogen activator and/or other protease(s) from the feedstock Impurities may be removed or reduced, for example, by precipitating fibrinogen and/or Factor VIII and/or VWF from the feedstock and recovering the precipitated protein(s). Suitable methods of precipitating fibrinogen and/or Factor VIII and/or VWF from a feedstock comprising fibrinogen and/or Factor VIII and/or VWF will be known to persons skilled in the art. An example includes adding aluminium hydroxide suspension to the feedstock, which is particularly useful for removing vitamin K-dependent proteins (for example, the clotting factors II, VII, IX, and X) and other proteins that have binding affinity for aluminium hydroxide, such as prothrombin (factor II) and t-PA, from plasma or plasma cryoprecipitate.

[0131] Thus, in an embodiment disclosed herein, prior to step (i), vitamin K-dependent proteins are removed or reduced from the feedstock. In a further embodiment, vitamin K-dependent proteins are removed or reduced by addition of aluminium hydroxide to the feedstock. Aluminium hydroxide may be in the form of Alhydrogel®, added to the feedstock to a final concentration of about 10% to about 80% w/w. In some embodiments, the aluminium hydroxide is added to the feedstock to a final concentration in the range from about 10% to about 50% (w/w). In preferred embodiments, the concentration is from about 15% to about 30% (w/w). Most preferably, the aluminium hydroxide is added to the feedstock at about 20% to about 25% (w/w) for optimum fibrinogen recovery and removal of impurities such as prothrombin. In another embodiment, vitamin K-dependent proteins are removed from the feedstock by batch adsorption using aluminium hydroxide.

[0132] In another aspect of the present invention, there is provided a solution comprising fibrinogen and/or Factor VIII and/or VWF that is recovered by the method of the present invention, as herein described. In an embodiment, the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution will be less than 20% of total protein, preferably less than 10% of total protein, and more preferably less than 5% of total protein. The skilled person will understand that the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) that is present in the solution comprising fibrinogen and/or Factor VIII and/or VWF may depend on the intended use of the solution or length of storage. For example, where the solution is to be stored for at least 4 weeks at a temperature of about 0° C. to about 8° C., it may be acceptable that the solution comprises more than about 10% plasminogen and/or tissue plasminogen activator and/or other protease(s) (of total protein). Where the solution is to be stored for at least 4 weeks at a temperature of about 30° C., it may be desirable that the solution comprises less than about 10% plasminogen and/or tissue plasminogen activator and/or other protease(s) (of total protein).

[0133] In an embodiment disclosed herein, there is provided a solution comprising fibrinogen recovered by the method of the present invention. In another embodiment, the solution comprises at least 80% total protein of fibrinogen.

[0134] In another aspect of the present invention, there is provided a solution comprising:

[0135] (a) at least 75% total protein of fibrinogen;

[0136] (b) less than 50 pg/mg total protein of tissue plasminogen activator; and

[0137] (c) less than 1 μg/mg total protein of plasminogen.

[0138] In an embodiment, the solution further comprises less than 1.5×10^{-5} U/mg total protein of Factor II.

[0139] In another aspect of the present invention, there is provided a solution comprising:

[0140] (a) at least 90% total protein of fibrinogen;

[0141] (b) less than 50 pg/mg total protein of tissue plasminogen activator; and

[0142] (c) less than 150 ng/mg total protein of plasminogen.

[0143] In an embodiment, the solution further comprises:

[0144] (a) less than 3.5×10^{-6} U/mg total protein of Factor II; and/or

[0145] (b) less than 150 μg/mg total protein of fibronectin.

[0146] In another aspect of the present invention, there is provided a solution comprising:

[0147] (a) at least 90% total protein of fibrinogen;

[0148] (b) less than 20 pg/mg total protein of tissue plasminogen activator; and

[0149] (c) less than 10 ng/mg total protein of plasminogen.

[0150] In an embodiment, the solution further comprises:

[0151] (a) less than 2.7×10^{-6} U/mg total protein of Factor II; and/or

[0152] (b) less than 15 μg/mg total protein of fibronectin.

[0153] The concentration of the fibrinogen and/or Factor VIII and/or VWF in the solution recovered by the methods disclosed herein and the concentration of impurities (e.g., plasminogen and/or tissue plasminogen activator and/or other protease(s)) can be measured by any means known to persons skilled in the art. Examples of suitable assays for measuring fibrinogen are described by Mackie et al. (*Br J Haematol.* 2003 May; 121(3):396-404). Size exclusion HPLC may also be used to measure the concentration of fibrinogen and/or Factor VIII or an impurity in the solution comprising fibrinogen and/or Factor VIII (e.g., Cardinali et al. 2010, Arch. Biochem. Biphys. 493(2):157-168; and Kosloski et al. 2009, AAPS J. 11(3); 424-431). HPLC also allows the skilled person to discriminate between monomers and aggregates of fibrinogen. Moreover, the concentration of fibrinogen and/or Factor VIII and/or VWF may differ depending on the sensitivity of the assay that is used. For instance, the concentration of fibrinogen in a solution as measured using the Clauss assay may be slightly lower than the concentration as measured in the same solution by HPLC.

[0154] In an embodiment disclosed herein, the concentration of monomeric fibrinogen in the solution is at least 75%, at least 80%, at least 90% or at least 95% of total protein as measured by size exclusion HPLC.

[0155] In another aspect of the present invention, there is provided a pharmaceutical formulation comprising a solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the methods disclosed herein, and a pharmaceutically acceptable carrier. Suitable pharmaceutically acceptable carriers, including pharmaceutically acceptable diluents and/or excipients, will be known to those skilled in the art. Examples include solvents, dispersion media, antifungal and antibacterial agents, surfactants, isotonic and absorption agents and the like.

[0156] The pharmaceutical formulation may also be formulated by the addition of a combination of suitable stabilisers, for example, an amino acid, a carbohydrate, a salt, and a detergent. In particular embodiments, the stabiliser comprises a mixture of a sugar alcohol and an amino acid. The stabilizer may comprise a mixture of a sugar (e.g. sucrose or trehalose), a sugar alcohol (e.g. mannitol or sorbitol), and an amino acid (e.g. proline, glycine and arginine). In a preferred embodiment, the formulation comprises an amino acid such as arginine. In other embodiments, the formulation comprises divalent metal ions in a concentration up to 100 mM and a complexing agent as described in U.S. Pat. No. 7,045,601. Particular embodiments include formulations 1 to 7 as described in Example 1 of U.S. Pat. No. 7,045,601. In particular embodiments, the formulation is formulated without the addition of any anti-fibrinolytic agents or stabilising proteins such as albumin. In embodiments where the formulation comprises fibrinogen, the pH is preferably about 6.5 to 7.5 and the osmolality is at least 240 mosmol/kg.

[0157] The pharmaceutical formulation may also be sterilised by filtration prior to dispensing and long term storage. Preferably, the formulation will retain substantially its original stability characteristics for at least 2, 4, 6, 8, 10, 12, 18, 24, 36 or more months. For example, formulations stored at 2-8° C. or 25° C. can typically retain substantially the same molecular size distribution as measured by HPLC-SEC when stored for 6 months or longer. Particular embodiments of the pharmaceutical formulation can be stable and suitable for commercial pharmaceutical use for at least 6 months, 12 months, 18 months, 24 months, 36 months or even longer when stored at 2-8° C. and/or room temperature.

[0158] The solutions and pharmaceutical formulations of the present invention, as herein described, may be formulated into any of many possible dosage forms, such as injectable formulations. The formulations and their subsequent administration (dosing) are within the skill of those in the art. Dosing is dependent on the responsiveness of the subject to treatment, but will invariably last for as long as the desirable effect is required (e.g., a return to normal plasma levels of fibrinogen). Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates.

[0159] In an embodiment disclosed herein, the pharmaceutical formulation of the present invention has a volume of at least 5 mL and comprises at least 5 mg/mL fibrinogen. In another embodiment, the pharmaceutical formulation has a volume of at least 5 mL and comprises at least 20 mg/mL fibrinogen. In particular embodiments, the pharmaceutical formulation has a volume of at least 5 mL and comprises fibrinogen at a concentration of about 20 mg/mL, 25 mg/mL, 30 mg/mL, 35 mg/mL, 40 mg/mL, 45 mg/mL, 50 mg/mL, 55 mg/mL, 60 mg/mL, 65 mg/mL, 70 mg/mL, 75 mg/mL, 80 mg/mL, 90 mg/mL or 100 mg/mL. In another aspect, there is provided a vessel containing at least 5 mL of a stable pharmaceutically acceptable fibrinogen solution, wherein the concentration of fibrinogen is at least 20 mg/mL.

[0160] In another aspect of the present invention, there is provided a method of treating or preventing a condition associated with fibrinogen and/or Factor VIII and/or VWF deficiency, the method comprising administering to a subject in need thereof a solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the method of the present invention, as herein disclosed, or the pharmaceutical formulation of the present invention, as herein disclosed.

[0161] In another aspect of the present invention, there is provided use of a solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the method of the present invention, as herein disclosed, in the manufacture of a medicament for treating or preventing a condition associated with fibrinogen and/or Factor VIII and/or VWF deficiency. Persons skilled in the art will be familiar with the types of conditions associated with fibrinogen and/or Factor VIII and/or VWF deficiency. In an embodiment, the fibrinogen condition is selected from the group consisting of afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia. In an embodiment the Factor VIII and/or VWF condition is selected from the group consisting of hemophilia A, bleeding disorders (e.g., defective platelet function, thrombocytopenia or von Willebrand's disease), vascular injury, bleeding from trauma or surgery, bleeding due to anticoagulant therapy, bleeding due to liver disease.

[0162] Other conditions that may be treatable with a solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the methods of the present invention, as herein disclosed, include major wounds and severe haemorrhaging and burns. In cases of hypofibrinogenamia and afibrinogenemia, a solution comprising fibrinogen prepared in accordance with the present invention can be injected intravenously into the patient in need thereof in order to compensate the state of fibrinogen deficiency and dosages can be determined by the skilled person based on the degree of deficiency.

[0163] A solution comprising fibrinogen recovered by the methods of the present invention also has advantages in the use of fibrin glues (also known as fibrin sealants) due to the lack of destabilizing levels of plasminogen and/or tissue plasminogen activator and/or other protease(s). tPA converts plasminogen to its active form plasmin which in turn digests the fibrin clot and therefore reduces clot formation upon topical application (e.g. for haemostasis).

[0164] Fibrin glues typically comprise two components: (i) fibrinogen (frequently together with factor XIII and a fibrinolysis inhibitor such as aprotinin), and (ii) thrombin (frequently together with calcium ions). The two components are reconstituted in order to prepare the glue ready for use. Fibrin glue is used in clinical and veterinary applications to simulate the last step of coagulation through the formation of cross-linked fibrin fibres using a combination of fibrinogen with thrombin in the presence of calcium and Factor XIII. Fibrin glue has diverse applications in clinical and veterinary medicine, including haemostasis, wound closure, adhesion prophylaxis and wound healing. Fibrin glue can also be used to close skin wounds (including skin transplant), for sealing sutures and for bonding connective tissues, such as bone, cartilage and tendons. Thus, in another aspect disclosed herein, there is provided a fibrin glue comprising the solution comprising fibrinogen recovered by the methods of the present invention, as herein disclosed.

[0165] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

[0166] Certain embodiments of the invention will now be described with reference to the following examples which are intended for the purpose of illustration only and are not intended to limit the scope of the generality hereinbefore described.

EXAMPLES

Example 1

Purification of Fibrinogen Through HEA, PPA and MEP Hydrophobic Charge Induction Chromatographic (HCIC) Resin

[0167] Human pooled plasma cryoprecipitate was used as the starting material (i.e., fibrinogen-containing feedstock). Briefly, the pooled plasma cryoprecipitate was solubilised in an extraction buffer containing 20 mM Tri-sodium citrate, 200 mM epsilon-amino caproic acid (ϵ-ACA), 60 IU/mL heparin and 500 mM NaCl (pH 7.2±2) at 31±2° C. for 30 minutes (1 g cryoprecipitate per 4 g of buffer). Aluminium hydroxide 2% (w/w) was then added to the solubilised cryoprecipitate at a concentration *of* 25% (w/w). After which the aluminium hydroxide gel was removed by either centrifugation or depth filtration and the fibrinogen-containing supernatant was recovered for further chromatographic purification through a HCIC chromatographic resin.

[0168] The fibrinogen-containing supernatant was applied to chromatography columns that were packed with 1.8 mL of either HEA, PPA or MEP Hypercel resin. The chromatography columns were pre-equilibrated in 25 mM Tris at a different pH, ranging from 6.5 to 8.5. The fibrinogen-containing supernatant was loaded onto the chromatography column at a ratio of approximately 11 mL/mL resin. HCIC purification was performed in negative mode with respect to fibrinogen, in which fibrinogen was allowed to drop-through in the unbound flow-through fraction, whilst the majority of t-PA, plasminogen and Factor II remained bound to the resin.

[0169] FIGS. **1** to **3** show step recovery of fibrinogen, plasminogen, t-PA and Factor II post-chromatographic purification using HEA Hypercel, PPA Hypercel and MEP Hypercel. The results show that pH has little or no effect on plasminogen binding to these resins, whereas the binding of t-PA to the resins appears to be most effective at the lower pH range. The HEA Hypercel column showed the highest fibrinogen recovery in the drop-through fraction and the operating pH range tested appeared to have little effect on fibrinogen recovery compared to that observed for both PPA and MEP Hypercel columns Both PPA and MEP columns displayed highest fibrinogen recovery in the drop-through fraction at pH 8.5.

Example 2

Level of Impurities in a Fibrinogen Solution Reduced Through HEA Hypercel

[0170] Approximately 48.5 mL of fibrinogen containing solubilised cryoprecipitate prepared according to Example 1 was applied onto a 5 mL HEA Hypercel column which was pre-equilibrated in 25 mM Tris at either pH 6.5, 7.0, 7.5, 8.0 or 8.5. HCIC purification was performed in negative mode with respect to fibrinogen, in which fibrinogen was allowed to drop-through in the unbound flow-through fraction, whilst t-PA, plasminogen and Factor II remained bound to the resin. FIG. **4***a* shows step recovery of fibrinogen, plasminogen, t-PA and Factor II during post-chromatographic purification using HEA Hypercel. The results show that pH had little or no effect on the binding of plasminogen and Factor II to the HCIC resin, whereas the binding of t-PA to the resin appeared to be most effective at the lower pH range *of* 6.5-7.0. Recovery of fibrinogen was greater than 90% in the drop-through fraction under different pH conditions despite no column wash being performed. As shown in FIG. **4***a*, these results demonstrate effective removal of proteases in a crude fibrinogen-containing feed stock, such as solubilised cryoprecipitate, by the HCIC resin. For example, the experimental condition performed at pH 7.0 demonstrated a reduction *of* >99.9% for factor II, 88.3% for t-PA and >98.2% for plasminogen from the solubilised cryoprecipitate solution.

[0171] The fibrinogen remained stable in solution for at least 6 days at room temperature (approx. 20° C.). The results are presented in FIGS. **4***b* and **4***c*.

Example 3

Level of Impurities in a Fibrinogen Solution Purified Through HEA Hypercel

[0172] Approximately 500 mL of the fibrinogen-containing supernatant obtained post alhydrogel adsorption step generated in accordance with Example 1 was loaded onto an XK 16/30 column packed with 36 mL of HEA Hypercel resin, pre-equilibrated in 25 mM Tris pH 7.0. The drop-through fraction was collected for fibrinogen, plasminogen, t-PA, and Factor II testing. A summary of the results is provided in Table 1 below.

TABLE 1

	Volume (mL)	Protein (mg/mL)	Fibrinogen by Clauss (mg/mL)	Plasminogen (ng/mL)	t-PA (ρg/mL)	Factor II (U/mL)
Fibrinogen supernatant	500	21.8	16.0	53172.0	1716.5	0.00097
Drop-through fraction	540	17.6	13.2	15655.5	706.7	0.00025
% recovery in drop-through fraction		87.2%	89.1%	31.8%	44.5%	27.8%

Example 4

The Effect of Glycine Precipitation on the Level of Impurities in a Fibrinogen Solution Purified Through HEA Hypercel

[0173] The fibrinogen-containing supernatant post-alhydrogel adsorption step generated in accordance with Example 1 was subjected to a further precipitation step by adding a saline solution comprising 2.4M glycine, 2.7M NaCl, 2.1 mM $CaCl_2$ and 23 mM Tri-sodium citrate (pH 6.6-7.3). The solubilised precipitate was warmed to 30° C. before being added to the glycine buffer, which was also incubated to 30° C., at a product to buffer ratio *of* 1:2. The mixture was allowed to stir for 10 minutes and the resultant precipitate was recovered from the liquid phase by centrifugation. The liquid phase, which contained predominantly fibronectin and IgG, was discarded and the fibrinogen-containing precipitate was collected and resuspended in a solubilisation buffer containing 100 mM NaCl, 1.1 mM $CaCl_2$, 10 mM Trisodium citrate, 10 mM Tris-(hydroxymethyl methylamine) and 4.5 mM sucrose (pH 7.0). The solubilised fibrinogen intermediate (250 mL) was clarified using a 1 μm filter before being passed through an XK 16/30 column packed with 36 mL of HEA Hypercel resin pre-equilibrated in 25 mM Tris pH 7.0. The drop-through fraction was collected for fibrinogen, plasminogen, t-PA and Factor II testing and a summary of the results is provided in Table 2 below.

[0174] The results demonstrate that binding of plasminogen, t-PA and Factor II to the HEA Hypercel resin was more effective under these processing conditions in comparison to that observed under the conditions described in Example 2. The characterisation results show a step recovery of approximately 93% for fibrinogen, 6% for plasminogen, 12% for t-PA and 5% for Factor II.

TABLE 2

	Volume (mL)	Protein (mg/mL)	Fibrinogen by Clauss (mg/mL)	Plasminogen (ng/mL)	t-PA (ρg/mL)	Factor II (U/mL)
Solubilised fibrinogen intermediate	250.0	29.7	30.3	48457.0	3408.4	0.002
Drop-through fraction	285.2	26.4	24.6	2646.8	984.2	0.00008
% recovery in drop-through fraction		101%	92.6	6.2%	12.3%	4.6%

Example 5

Preparation of Purified Fibrinogen from Plasma Cryoprecipitate

[0175] Process Step 1—solubilisation of plasma cryoprecipitate;

[0176] Process Step 2—Alhydrogel (aluminium hydroxide) adsorption (Alhydrogel concentration: target 20% w/w, ranging from 15 to 50% w/w) of solubilised plasma cryoprecipitate and recovery of fibrinogen-containing supernatant using methods such as centrifugation or depth filtration in the presence of a filter aid. Alternatively this step can be replaced with either a HCIC chromatographic step in negative mode (drop through) with respect to fibrinogen or a combination of HCIC and anion exchange chromatography both in negative mode with respect to the fibrinogen. If both HCIC and anion exchange chromatography are used in combination then Process Step 3 is optional;

[0177] Process Step 3—glycine precipitation of fibrinogen from the fibrinogen-containing supernanant of Step 2. Alternatively this step can be replaced with an anion exchange chromatography in negative mode with respect to the fibrinogen.

[0178] Process Step 4—passing the solubilised glycine precipitate from Step 3 through a HCIC chromatographic resin in negative mode with respect to fibrinogen;

[0179] Process Step 5—treating the purified fibrinogen solution recovered in Step 4 with solvent or detergent or pasteurisation to inactivate pathogens.

[0180] Process Step 6—passing the treated solution from Step 5 through an anion exchange chromatographic resin in positive mode with respect to fibrinogen, washing the weakly bound proteins from the resin and eluting the fibrinogen from the resin;

[0181] Process Step 7—subjecting the fibrinogen eluted from the anion exchange resin in Step 6 to nanofiltration (35 nm or 20 nm or a combination *of* 35/20 nm); and

[0182] Process Step 8—subjecting the filtered fibrinogen from Step 7 to ultrafiltration (50, 100, 200 and 300 kDa membrane filters).

Example 6

Preparation of Purified Fibrinogen by Combination of HCIC and Anion Exchange Chromatography

[0183] Three laboratory scale experiments were completed in which approximately 100 g of cryoprecipitate was prepared according to the steps described in Examples 1 to 3 through to the mixed mode chromatography step using a HEA Hypercel column.

[0184] The drop-through fraction from the HEA Hypercel column, which contained predominantly fibrinogen, was subjected to an overnight solvent/detergent treatment for virus inactivation.

[0185] The virus inactivated solution was then diluted to <10 mS/cm using 25 mM Tris (pH 8.0) prior to being loaded onto an anion exchange column (XK 50/30, GE Healthcare), packed with approximately 412 mL of Macroprep-HQ resin that was pre-equilibrated with 25 mM Tris (pH 8.0). The flow-though fraction was discarded and the Macroprep-HQ column washed with 4 column volumes of a washing buffer containing 90 mM NaCl, 50 mM Tris, 20 mM EACA (pH 8.0). Under these chromatographic conditions, the initial flow-through fraction and the wash fractions contain predominantly plasminogen and t-PA, whilst fibrinogen remained bound to the chromatographic resin. The monomeric form of fibrinogen was selectively eluted from the Macroprep-HQ column using an elution buffer comprising 200 mM NaCl, 10 mM Tris, 10 mM Tri-sodium citrate, 46 mM sucrose and 1.1 mM $CaCl_2$ (pH 7.0), leaving fibrinogen aggregates and low molecular weight proteins bound to the Macroprep-HQ resin.

[0186] Product intermediates generated from solubilised cryoprecipitate through to Macroprep-HQ chromatography eluate were characterised for fibrinogen, t-PA, plasminogen, fibronectin and Factor II levels and process step recoveries for each of these proteins at the various process stages. The results are shown in Table 3, which represent a mean value from three separate laboratory scale consistency batches. The overall process recovery for fibrinogen and co-purified proteins starting from the plasma cryoprecipitate through to the Macroprep-HQ eluate is shown in FIG. **5**.

[0187] The purity of fibrinogen that was recovered from the Macroprep-HQ chromatographic resin was greater than 95%, as revealed by analytical size exclusion HPLC chromatography. A representative of the HPLC profile of fibrinogen analysed on TSKGel G4000SWXL (Tosoh Corporation) is shown in FIG. **6**. As shown in FIG. **6**, the fibrinogen monomer is eluted at a retention time of approximately 17.4 minutes and contributed to 96.6% of the total peak area, whilst fibrinogen dimer and/or other high molecular weight proteins are eluted at a retention time of approximately 14.8 minutes.

Example 7

Stability Study

[0188] Purified fibrinogen solution recovered by the method described in Example 5 above was sterile filtered and subjected to a stability study over a 9/7 week period at 2°-8° C. or 30° C. The liquid fibrinogen preparation placed at 2°-8° C. retained from about 90% of its original activity as measured by the Clauss method after the 9 week storage period. The liquid fibrinogen preparation placed at 30° C. retained about 70% of its original activity after the 2 week storage period and did not lose further activity for at least 5 weeks. A further reduction in activity to below 60% was observed by week 7 of incubation at 30° C. The loss of fibrinogen activity at 30° C. is likely to be attributed to heat denaturation, rather than proteolytic degradation, as the addition of a protease inhibitor (C1 Esterase) did not inhibit the loss of fibrinogen activity over the 5 week storage period. A summary of the stability data is provided in FIG. **7**.

TABLE 3

	Protein mg/mL) (n = 3)		Clottable protein (n = 3)		Plasminogen (n = 3)		t-PA (n = 3)		Factor II (n = 3)		Fibronectin (n = 3)	
Processing steps	Conc. (mg/mL)	Step recovery (%)	Conc. (mg/mL)	Step recovery (%)	Conc. (ug/mL)	Step recovery (%)	Conc. (ng/mL)	Step recovery (%)	Conc. (U/mL)	Step recovery (%)	Conc. (mg/mL)	Step recovery (%)
Solubilised cryoprecipitate	31.0		23.5		65.44		9.00		0.28041		8.15	
Solubilised cryoprecipitate filtrate-post Al(OH)3 treatment	15.8	81%	11.6	80%	39.04	96%	2.20	39%	0.00057	0.3%	3.47	70%
Solubilised glycine saline precipitate	30.3	76%	27.6	95%	45.78	46%	5.55	101%	0.00025	26.0%	3.18	37%
HEA Hypercel drop-through fraction	22.7	93%	20.5	92%	2.20	6%	0.98	22%	0.00008	39%	2.79	97%
SD Incubation & Macroprep-HQ chromatography	7.4	80%	6.7	79%	0.04	5%	0.13	38%	<0.00002	0%	0.09	8%

1. A method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or von Willebrand factor (VWF), the method comprising:

(i) passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a hydrophobic charge-induction chromatographic resin under conditions selected

such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the resin; and

(ii) recovering a solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the resin;

wherein the concentration of plasminogen and/or tissue plasminogen activator and/or proteases(s) in the recovered solution is reduced by at least 50% compared to the feedstock.

2. A method of reducing the level of plasminogen and/or tissue plasminogen activator and/or other protease(s) in a solution comprising fibrinogen and/or Factor VIII and/or von Willebrand factor (VWF), the method comprising:

(i) passing a feedstock comprising fibrinogen and/or Factor VIII and/or VWF through a first hydrophobic charge-induction chromatographic resin;

(ii) recovering a solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the first hydrophobic charge-induction chromatographic resin;

(iii) passing the solution that is recovered in step (ii) through a second hydrophobic charge-induction chromatographic resin; and

(iv) recovering a solution comprising fibrinogen and/or Factor VIII and/or VWF which passes through the second hydrophobic charge-induction chromatographic resin;

wherein the conditions of the chromatographic steps are such that the plasminogen and/or tissue plasminogen activator and/or other protease(s) is bound to the first and/or second resin, and wherein the concentration of plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution that is recovered in step (iv) is reduced by at least 50% compared to the feedstock.

3. The method of claim **2** wherein the first and second hydrophobic charge-induction chromatographic resins are the same.

4. The method of claim **1**, further comprising passing the solution comprising fibrinogen and/or Factor VIII and/or VWF recovered in step (ii) through an anion exchange chromatographic resin.

5. The method of claim **2**, further comprising passing the solution comprising fibrinogen and/or Factor VIII and/or VWF recovered in step (ii) and/or step (iv) through an anion exchange chromatographic resin.

6. The method of claim **1**, further comprising passing the feedstock comprising fibrinogen and/or Factor VIII and/or VWF through an anion exchange chromatographic resin prior to step (i).

7. The method of claim **4**, wherein the anion exchange resin is a strong anion exchange resin.

8. The method of claim **4**, wherein the solution comprising the fibrinogen and/or Factor VIII and/or VWF is passed through the anion exchange chromatographic resin in the presence of about 170 mM to about 230 mM NaCl.

9. The method of claim **4**, wherein fibrinogen is eluted from the anion exchange chromatographic resin with an elution buffer comprising from about 150 mM to about 300 mM NaCl.

10. The method of claim **7**, wherein fibrinogen is eluted from the anion exchange chromatographic resin with an elution buffer comprising a free amino acid at a concentration of about 1-3% w/v.

11. The method of claim **10**, wherein the free amino acid is arginine.

12. The method of claim **1**, wherein the feedstock comprising fibrinogen and/or Factor VIII and/or VWF is subjected to a viral inactivation step prior to step (i).

13. The method of claim **1**, wherein the solution comprising fibrinogen and/or Factor VIII and/or VWF recovered from the hydrophobic charge-induction chromatographic resin in step (ii) is subjected to a viral inactivation step.

14. The method of claim **4**, wherein the feedstock or the solution comprising fibrinogen and/or Factor VIII and/or VWF recovered in step (ii) is subjected to a viral inactivation step before it is passed through the anion exchange chromatographic resin.

15. The method of claim **11**, wherein the viral inactivation step comprises pasteurisation or treatment with an organic solvent and detergent.

16. The method of claim **1**, wherein the feedstock comprising fibrinogen and/or Factor VIII and/or VWF is a solubilised plasma cryoprecipitate.

17. The method of claim **1**, wherein, prior to step (i), vitamin K-dependent proteins are removed or reduced from the feedstock.

18. The method of claim **17**, wherein the vitamin K-dependent proteins are removed or reduced by precipitating the vitamin K-dependent proteins from the feedstock by adding aluminium hydroxide to the feedstock.

19. The method of claim **1**, further comprising, prior to step (i), precipitating the fibrinogen and/or Factor VIII and/or VWF from the feedstock by adding glycine to the feedstock, recovering the precipitated fibrinogen and/or Factor VIII and/or VWF, solubilising the precipitated fibrinogen and/or Factor VIII and/or VWF, wherein the solubilised fibrinogen and/or Factor VIII and/or VWF is passed through the hydrophobic charge-induction chromatographic resin in step (i).

20. The method of claim **1**, wherein the feedstock has a pH from about 6.5 to about 8.5.

21. The method of claim **1**, wherein the hydrophobic charge-induction chromatographic resin is equilibrated at a pH from about 6.5 to about 8.5 prior to passing the feedstock through the resin.

22. The method of claim **1**, wherein the hydrophobic charge-induction chromatographic resin comprises a ligand selected from mercaptoethylpyridine, n-hexylamine and phenylpropylamine.

23. The method of claim **22**, wherein the hydrophobic charge-induction chromatographic resin comprises n-hexylamine.

24. A solution comprising fibrinogen and/or Factor VIII and/or VWF recovered by the method of claim **1**.

25. The solution of claim **24**, wherein at least 80% of the total protein of the solution comprises fibrinogen.

26. A solution comprising fibrinogen, wherein:

(a) at least 75% of the total protein of the solution comprises fibrinogen;

(b) less than 50 pg/mg of the total protein comprises tissue plasminogen activator; and

(c) less than 1 μg/mg of the total protein comprises plasminogen.

27. The solution of claim **26**, wherein less than 1.5×10^{-5} U/mg of the total protein comprises Factor II.

28. The solution of claim **26**, wherein:

(a) at least 90% of the total protein comprises fibrinogen;

(b) less than 50 pg/mg of the total protein comprises tissue plasminogen activator; and

(c) less than 150 ng/mg of the total protein comprises plasminogen.

29. The solution of claim **28**, wherein:

(a) less than 3.5×10^{-6} U/mg of the total protein comprises Factor II; and/or

(b) less than 150 μg/mg of the total protein comprises fibronectin.

30. The solution of claim **26**, wherein:

(a) at least 90% of the total protein comprises fibrinogen;

(b) less than 20 pg/mg of the total protein comprises tissue plasminogen activator; and

(c) less than 10 ng/mg of the total protein comprises plasminogen.

31. The solution of claim **30**, wherein

(a) less than 2.7×10^{-6} U/mg of the total protein comprises Factor II; and/or

(b) less than 15 μg/mg of the total protein comprises fibronectin.

32. The solution of claim **25**, wherein at least 80% of the total protein comprises monomeric fibrinogen.

33. The solution of claim **26**, wherein the fibrinogen retains from about 90% to 100% activity after at least 4 weeks in storage at a temperature of about 0° C. to about 8° C.

34. The solution of claim **26**, wherein the fibrinogen retains from about 60% to about 70% activity after 5 weeks in storage at a temperature of about 30° C.

35. A pharmaceutical formulation comprising the solution of claim **26** and a pharmaceutically acceptable carrier.

36. The pharmaceutical formulation of claim **35** having a volume of at least 5 mL and comprising at least 5 mg/mL fibrinogen.

37. The pharmaceutical formulation of claim **35** having a volume of at least 5 mL and comprising at least 20 mg/mL fibrinogen.

38. A method of treating or preventing a condition associated with fibrinogen deficiency, the method comprising administering to a subject in need thereof the solution of claim **26**.

39. The method of claim **38**, wherein the condition is selected from afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia.

40.-**41**. (canceled)

42. A fibrin glue comprising the solution of claim **26**.

43. A method of producing a stable liquid fibrinogen solution, the method comprising:

(i) passing a feedstock comprising fibrinogen through a hydrophobic charge-induction chromatographic resin under conditions selected such that plasminogen and/or tissue plasminogen activator and/or other protease(s) are bound to the resin; and

(ii) recovering a solution comprising fibrinogen which passes through the resin;

wherein the concentration of plasminogen and/or tissue plasminogen activator and/or proteases(s) in the recovered solution is reduced by at least 50% compared to the feedstock.

44. A method of producing a stable liquid fibrinogen solution, the method comprising:

(i) passing a feedstock comprising fibrinogen through a first hydrophobic charge-induction chromatographic resin;

(ii) recovering a solution comprising fibrinogen which passes through the first hydrophobic charge-induction chromatographic resin;

(iii) passing the solution that is recovered in step (ii) through a second hydrophobic charge-induction chromatographic resin; and

(iv) recovering a solution comprising fibrinogen which passes through the second hydrophobic charge-induction chromatographic resin;

wherein the conditions of the chromatographic steps are such that plasminogen and/or tissue plasminogen activator and/or other protease(s) are bound to the first and/or second resin, and wherein the concentration of plasminogen and/or tissue plasminogen activator and/or other protease(s) in the solution that is recovered in step (iv) is reduced by at least 50% compared to the feedstock.

45. The method of claim **43**, wherein the stable liquid fibrinogen solution retains from about 90% to 100% activity after at least 4 weeks in storage at a temperature of about 0° C. to about 8° C.

46. The method of claim **43**, wherein the stable liquid fibrinogen solution retains from about 60% to about 70% activity after at least 5 weeks in storage at a temperature of about 30° C.

47. A vessel containing at least 5 mL of the stable liquid fibrinogen solution prepared according to claim **43**, wherein the concentration of fibrinogen is at least 20 mg/mL.

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