

PROCESS FOR PROTEIN ISOLATION

Field of the Invention

The present invention relates to processes for isolating proteins from biological sources. More particularly, the invention relates to processes for isolating proteins from blood.

Background of the Invention

Plasma is one of nature's most valuable raw materials and proteins purified from plasma are essential to the life and well-being of millions of individuals around the world. The majority of these proteins are produced by Cohn cold-ethanol fractionation. This process was developed by Dr Edwin Cohn at Harvard University in the early 1940s. Cohn found that it was possible to separate proteins in plasma based on their precipitation characteristics under different conditions (pH, ionic strength, protein concentration, temperature and ethanol concentration). By varying these parameters, different proteins precipitate out in a step by step fashion. Cohn technology has been used in the plasma industry for decades but the process presents some limitations in purity and efficiency. While this process can produce a quality product, there are limitations to the flexibility of the procedure and purity of the products produced. The most common commercially produced proteins still using this methodology include albumin, immunoglobulin, anti-thrombin III, thrombin and fibrinogen.

One type of technical advance in plasma product manufacture incorporates the use of chromatography, which separates the proteins in plasma on the basis of selective adsorption of the protein molecules onto a stationary solid surface (solid phase) as the liquid (mobile phase) percolates down an enclosed column containing the stationary phase. Depending on the efficiency of adsorption or interaction, the movement of various proteins is retarded to different extents, enabling their separation and collection from the bottom of the column.

Chromatography is essentially a more gentle means of separating proteins than the traditional Cohn cold-ethanol process, which involves prolonged exposure of the proteins to high concentrations of ethanol. This can denature the proteins and produce unwanted aggregates, which in turn have adverse therapeutic consequences to patients receiving these products. In contrast, chromatographic fractionation efficiently removes impurities without affecting the native structure of the proteins. Compared with the repeated large-scale precipitations involved in Cohn cold-ethanol fractionation, chromatography is a

more direct method of separation, enabling increased amounts of protein to be produced per litre of plasma.

The chromatographic methods which have been adopted to date still provide challenges in terms of optimizing yields while retaining or enhancing the necessary levels of purity. There remains a need for an efficient and cost effective chromatographic technique that selectively elutes plasma components at a superior purity and yield to existing methods.

In the early 1960's, it was noticed that when plasma was incubated at low temperatures, a precipitate was seen to form. This precipitate was established to contain Factor VIII, Von Willebrands Factor and a number of other plasma proteins. Initially this cryoprecipitate was used to treat Haemophilia A patients but with the need to improve patient tolerance for the product, more purified forms of Factor VIII became available. The process of cryoprecipitation remains the first basis of Factor VIII production used by industry today. Unfortunately the process is not highly efficient, and while various attempts have been made to improve recovery of Factor VIII by directly using plasma rather than cryoprecipitate, few of these attempts have been commercially successful.

Factor IX is used for the treatment of Haemophilia B patients and is isolated from supernatant I, a side fraction of the well established Cohn plasma fractionation process. The Factor IX in supernatant I is usually further purified with an affinity chromatography step using Heparin Sepharose in essentially all current manufacturing processes, however since the overall process is based on Cohn fractionation, significant limitations in manufacturing efficiency still remain.

In this case again, there is a need for more efficient processes for the isolation of Factor VIII and/or Factor IX from mixtures containing Factor VIII and/or Factor IX.

Summary of the Invention

According to a first aspect, the present invention provides a process for isolating proteins from a solution comprising said proteins, said solution being selected from the group consisting of: crude blood plasma, blood serum, cryosupernatant derived from plasma, fractionated human plasma, cryoprecipitate derived from plasma and recombinant broths, said process comprising:

- (i) providing a solid separation medium having the formula:

M-S-L

wherein M is a matrix backbone, S is an optional spacer arm, and L is a ligand which is mercaptonicotinic acid;

(ii) contacting said solid separation medium with said solution, such that at least one of said proteins becomes reversibly bound to said solid separation medium;

5 (iii) performing at least one elution step to selectively elute from the solid separation medium, at least one protein fraction.

The following features relate to the first aspect of the invention.

In one embodiment the proteins are of mammalian or human origin.

The spacer arm S may be derived from a compound comprising an epoxy group
10 (e.g. butane dioldiglycidylether or epichlorohydrin) or other suitable coupling reagent known in the art for covalent attachment of ligands.

The matrix backbone M may be a resin such that the solid separation medium is a resin functionalised with the ligand. Further, the resin may be any resin to which the ligand may be attached. Further still, the resin may be a high density resin suitable for
15 non-packed bed adsorption, such as for example fluidized bed adsorption and expanded bed adsorption, such as a highly cross-linked beaded agarose derivative based on 6% or 4% agarose, agarose-tungsten carbide conglomerate, an agarose stainless steel conglomerate, an agarose-quartz conglomerate, porous ceramic beads, porous zirconia beads, beads made of controlled pore glass and composite beads of porous inorganic
20 materials comprising organic polymers within their pores.

In one embodiment the ligand may be 2-mercaptonicotinic acid.

As the binding of the protein(s) to the ligand is reversible, the protein may be isolated from the solid separation medium under the appropriate elution conditions described below.

25 The solid separation medium may comprise a high density resin having a mean particle size in the range of about 10 micron to about 150 micron and a bead density in the range from about 1.5 g/ml to 15 g/ml, or alternatively a mean particle size in the range of about 10 micron to 120 micron and a bead density from about 2.0 g/ml to 15g/ml, or alternatively a mean particle size in the range from about 15 micron to 100 micron and a
30 bead density in the range from about 2.3 g/ml to 15 g/ml, or alternatively a mean particle size in the range from about 15 micron to 80 micron and a bead density in the range from about 3 g/ml to 15 g/ml.

Proteins isolated by the process of the first aspect may be selected from the group consisting of: immunoglobulin for example, IgG, IgA, IgM, IgD, or IgE, transferrin (Tf),
35 fibrinogen or a derivative thereof, plasma protease inhibitor such as an antithrombin, e.g.,

antithrombin III, blood pro-coagulation protein, blood anti-coagulation protein, cytokine, growth factor, albumin or a derivative thereof, thrombolytic agent, anti-angiogenic protein, insulin or a derivative thereof, α -1-proteinase inhibitor or a derivative thereof, such as α -1-antitrypsin, α -2-antiplasmin or a derivative thereof, C-1 esterase inhibitor, 5 apolipoprotein, HDL, Fibronectin or a derivative thereof, beta-2-glycoprotein I, plasminogen, plasmin, plasminogen activator, plasminogen inhibitor, urokinase or derivative thereof, streptokinase or a derivative thereof, inter- α -trypsin inhibitor, α -2-macroglobulin, amyloid protein, orosomucoid, ferritin, pre-albumin, GC-globulin, haemopexin and C3-complement.

10 The protein fractions may contain a single protein. Alternatively, the protein fractions may contain multiple proteins.

The process may comprise from 1 to 50, from 1 to 30, from 1 to 20, from 1 to 10, from 1 to 5 elution steps, from 1 to 3, or a single elution step.

In one embodiment respective eluants may have a pH in the range from about 4.0 to 15 about 9.0. Alternatively, respective eluants may have a pH in the range from about 4.0 to 8.5. Alternatively, respective eluants may have a pH in the range from about 4.0 to about 8.0. Further, respective eluants may have a pH in the range from about 5.0 to about 8.0. Further, respective eluants may have a pH in the range from about 4.5 to 8.0. Further still, respective eluants may have a pH in the range from about 5.5 to about 8.0. Yet 20 further still respective eluants may have a pH in the range from about 6.0 to 8.0.

Respective eluants may have an ionic strength in the range from about 0.00005 Siemens/centimetre (S/cm) to about 10.0 S/cm. Alternatively, respective eluants may have an ionic strength in the range from about 0.0005 S/cm to 10.0 S/cm. Alternatively, respective eluants may have an ionic strength in the range from about 0.0001 S/cm to 25 about 6.0 S/cm. Further, respective eluants may have an ionic strength in the range from about 0.001 S/cm to about 5.5 S/cm. Further still, respective eluants may have an ionic strength in the range from about 0.001 S/cm to about 5.0 S/cm. Further still, respective eluants may have an ionic strength in the range from about 0.005 S/cm to about 5.0 S/cm. Yet further still the eluants may have an ionic strength in the range from about 0.01 S/cm 30 to about 4.0 S/cm.

Respective eluants may have any combinations of pH and ionic strength values within the above specified ranges.

The first, second or third eluants may have a pH of between about 4.0 and about 9.0, and an ionic strength of between about 0.00005 S/cm and about 10.0 S/cm. The first 35 eluant may have a pH of between about 4.0 and about 8.0 and an ionic strength of

between about 0.00005 S/cm and about 0.1 S/cm. Further, the first eluant may have a pH of between about 4.5 and about 6.5 and an ionic strength of between about 0.00005 S/cm and about 0.075 S/cm. Further the first eluant may have a pH of between about 5.0 and about 6.0 and an ionic strength of between about 0.001 S/cm and about 0.05 S/cm.

5 The second eluant may have a pH of between about 5.0 and about 7.0 and an ionic strength of between about 0.0001 S/cm and about 0.1 S/cm. Alternatively the second eluant may have a pH of between about 5.5 and about 6.5 and an ionic strength of between about 0.0001 S/cm and about 0.075 S/cm. Further the second eluant may have a pH of between about 5.5 and about 6.5 and an ionic strength of between about 0.001 S/cm and about 0.05 S/cm.

10 The third eluant may have a pH of between about 5.0 and about 9.0 and an ionic strength of between about 0.0001 S/cm and about 4.0 S/cm. Alternatively the third eluant may have a pH of between about 6.0 and about 8.0 and an ionic strength of between 0.01 S/cm and about 3.0 S/cm. Further the third eluant may have a pH of between about 6.0 and about 8.0 and an ionic strength of between about 0.05 S/cm and about 2.0 S/cm.

The first eluant may be an eluant that results in no loss of the biological function of the proteins, such as demineralised water or an aqueous solution of one or more inorganic salts of strong mineral acids, for example salts of hydrochloric acid, sulfuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulfate, potassium sulfate and ammonium sulfate. Alternatively, the first eluant may be an aqueous buffer solution comprising a salt of an inorganic and/or an organic acid which does not result in loss of the biological function of the proteins, for example a buffer comprising a citrate, an acetate, a succinate, a lactate, a tartrate, a formate, a propionate, a phosphate or a borate.

25 The second eluant may be an aqueous buffer solution comprising a salt of an inorganic and/or an organic acid that results in no loss of the biological function of the proteins, for example a buffer comprising a citrate, an acetate, a succinate, a lactate, a tartrate, a formate, a propionate, a phosphate or a borate. In one embodiment, the second eluant comprises a negatively charged molecule having a non-aromatic, an aromatic or a heteroaromatic hydrophobic moiety, such as salts of medium to long chain alkyl-carboxylic and alkyl-sulfonic acids, and negatively charged detergents, such as sodium dodecyl sulphate and sodium deoxycholate. For example, the second eluant may comprise a salt of one or more acids selected from the group consisting of: caproic acid, heptanoic acid, caprylic acid, perlagonic acid and capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid and pentadecanoic acid, and also unsaturated and

alkyl substituted derivatives thereof. Alternatively, the second eluant may comprise a salt of one or more acids selected from the group consisting of: hexane sulfonic acid, octane sulfonic acid, decane sulfonic acid, dodecane sulfonic acid, hexane sulfate, octane sulfate, decane sulfate and dodecane sulfate.

5 The third eluant may be an aqueous buffer solution comprising a salt of an inorganic and/or an organic acid that results in no loss of the biological function of the proteins, for example a buffer comprising a citrate, an acetate, a succinate, a lactate, a tartrate, a formate, a propionate, a phosphate, or a borate. Alternatively the third eluant may comprise an inorganic or organic salt of a relatively high lytrophobicity such as
10 ammonium sulfate, sodium sulfate, potassium sulfate, ammonium phosphate, sodium phosphate, potassium phosphate, ammonium citrate, sodium citrate, potassium citrate.

The process of the first aspect may comprise eluting the solid separation medium with a fourth eluant to selectively elute a fourth protein fraction. The fourth eluant may have a pH of between about 5.0 and about 9.0 and an ionic strength of between about 0.01
15 S/cm and about 2 S/cm. Alternatively the fourth eluant may have a pH between about 6.0 and about 8.0 and an ionic strength between about 0.01 S/cm and about 1.0 S/cm. Further, the fourth eluant may have a pH of between about 6.0 and about 8.0 and an ionic strength between about 0.05 S/cm and about 1.0 S/cm. The fourth eluant may be an aqueous buffer solution comprising a salt of an inorganic and/or an organic acid
20 compatible with the proteins to be isolated, for example a buffer comprising a citrate, an acetate, a succinate, a lactate, a tartrate, a formate, a propionate, a phosphate, or a borate.

The fourth eluant may also comprise an aqueous solution of one or more inorganic salts of mineral acids, in particular strong mineral acids, that result in no loss of the biological function of the proteins, for example salts of hydrochloric acid, sulphuric acid
25 and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulphate, potassium sulphate, ammonium sulphate.

The first protein fraction may comprise one or more of the following proteins selected from the group consisting of: albumin, orosomucoid, pre-albumin, α -1-proteinase inhibitor (α -1-PI), transferrin and fibrinogen.

30 The second protein fraction may comprise one or more of the following proteins selected from the group consisting of: antithrombin, e.g., Antithrombin III, albumin, immunoglobulins, transferrin and fibrinogen.

The third protein fraction may comprise one or more of the following proteins selected from the group consisting of: immunoglobulins, e.g., IgA, IgD, IgE, IgG and/or
35 IgM, transferrin and fibrinogen.

The fourth protein fraction may comprise one or more of the following proteins selected from the group consisting of: transferrin, α -2-macroglobulin, immunoglobulins such as IgM, and fibrinogen.

According to a second aspect, the present invention provides a process for isolating Factor VIII and/or Factor IX from a solution comprising Factor VIII and/or Factor IX, said solution being selected from the group consisting of: crude blood plasma, blood serum, cryosupernatant derived from plasma, fractionated human plasma, cryoprecipitate derived from plasma and recombinant broths, said process comprising:

- (i) providing a solid separation medium having the formula:

M-S-L

wherein M is a matrix backbone, S is an optional spacer arm and L is a ligand which is xylylenediamine;

- (ii) contacting said solid separation medium with said solution such that at least Factor VIII and/or Factor IX become reversibly bound to said solid separation medium;

- (iii) performing a first elution step to elute non-bound proteins from the solid separation medium;

- (iv) performing a second elution step to elute Factor VIII and/or Factor IX from the solid separation medium.

M may be a high density resin suitable for non-packed bed adsorption, for example fluidized bed adsorption and expanded bed adsorption, for example a highly cross-linked beaded agarose derivative based on 6% or 4% agarose, an agarose-tungsten carbide conglomerate, an agarose stainless steel conglomerate, an agarose-quartz conglomerate, porous ceramic beads, porous zirconia beads, beads made of controlled pore glass and composite beads of porous inorganic materials comprising organic polymers within their pores.

The high density resin may have a mean particle size in the range of about 10 micron to about 300 micron, or from about 15 micron to 150 micron, and a bead density in the range from about 1.1 g/ml to 15 g/ml, or from about 1.5 g/ml to 15 g/ml, or alternatively a mean particle size in the range of about 10 micron to 120 micron and a bead density from about 2.0 g/ml to 15 g/ml, or alternatively a mean particle size in the range from about 15 micron to 100 micron and a bead density in the range from about 2.3 g/ml to 15 g/ml, or alternatively a mean particle size in the range from about 15 micron to 80 micron and a bead density in the range from about 3 g/ml to 15 g/ml.

The spacer arm S may be derived from a compound comprising an epoxy group (e.g. butane dioldiglycidylether or epichlorohydrin) or other suitable coupling reagent known in the art for covalent attachment of ligands.

In one embodiment L may be *m*-xylylenediamine. Alternatively L may be *p*-xylylenediamine. L may also be *o*-xylylenediamine.

Non-bound proteins may be selected from, but not limited to, the group consisting of: IgG, IgA, IgM, IgD, or IgE, transferrin (Tf), fibrinogen or a derivative thereof, plasma protease inhibitor such as an antithrombin, e.g., antithrombin III, blood pro-coagulation protein, blood anti-coagulation protein, cytokine, growth factor, albumin or a derivative thereof, thrombolytic agent, anti-angiogenic protein, insulin or a derivative thereof, α -1-proteinase inhibitor or a derivative thereof, such as α -1-antitrypsin, α -2-antiplasmin or a derivative thereof, C-1 esterase inhibitor, apolipoprotein, HDL, Fibronectin or a derivative thereof, beta-2-glycoprotein I, plasminogen, plasmin, plasminogen activator, plasminogen inhibitor, urokinase or derivative thereof, streptokinase or a derivative thereof, inter- α -trypsin inhibitor, α -2-macroglobulin, amyloid protein, orosomucoid, ferritin, pre-albumin, GC-globulin, haemopexin and C3-complement.

The eluants used in the elution steps may have a pH in the range of about 5.0 to about 9.0. The eluants may have a conductivity in the range from about 0.0001 S/cm to about 10.0 S/cm. The eluants may have a pH in the range of about 6.0 to about 9.0, and a conductivity of about 0.03 S/cm to about 0.2 S/cm.

The eluants may have approximately the same pH but may differ in ionic strength. For example, respective eluants may comprise different buffer systems, and/or optionally comprise additional salts, for example salts of hydrochloric acid, sulphuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulphate, potassium sulphate, ammonium sulphate.

Respective eluants may have any combinations of pH and ionic strength values within the above specified ranges. The eluant may comprise an aqueous buffer solution comprising a salt of an inorganic or organic acid that results in no loss of the biological function of the Factor VIII and/or Factor IX, for example a buffer comprising a citrate, an acetate, a succinate, a lactate, a tartrate, a formate, a propionate, a borate or a phosphate. The eluant may also comprise an aqueous solution of one or more inorganic salts of strong mineral acids that result in no loss of the biological function of the proteins, for example salts of hydrochloric acid, sulphuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulphate, potassium sulphate, ammonium sulphate.

The first and second eluants may have a pH of between about 6.0 and about 8.0, and an ionic strength of between about 0.0001 S/cm and about 1.0 S/cm. Alternatively, the first eluant may have a pH of between about 5.5 and about 6.0 and an ionic strength of between about 0.0001 S/cm and about 0.01 S/cm.

5 Further, the first eluant may have a pH of between about 5.5 and about 6.0 and an ionic strength of between about 0.0001 S/cm and about 0.05 S/cm. Further the first eluant may have a pH of between about 6.0 and about 6.5 and an ionic strength of between about 0.001 S/cm and about 0.1 S/cm.

10 The second eluant may have a pH of between about 6.0 and about 8.0 and an ionic strength of between about 0.0001 S/cm and about 0.1 S/cm. Alternatively the second eluant may have a pH of between about 6.0 and about 8.0 and an ionic strength of between about 0.0001 S/cm and about 0.5 S/cm. Further the second eluant may have a pH of between about 7.0 and about 9.0 and an ionic strength of between about 0.001 S/cm and about 1 S/cm.

15 By controlling the pH and conductivity of the eluants as described in the above paragraphs, the process may be used to isolate Factor VIII and Factor IX as a mixture, from a solution comprising Factor VIII and Factor IX as well as other substances. Alternatively, where the solution comprises only Factor VIII (for example where the solution is cryoprecipitate derived from plasma, or a recombinant broth where only Factor
20 VIII has been expressed), by controlling the pH and conductivity of the eluants as described in the above paragraph, the process may be used to isolate only Factor VIII. Where the solution comprises only Factor IX (for example where the solution is cryosupernatant derived from plasma, or a recombinant broth where only Factor IX has been expressed), by controlling the pH and conductivity of the eluants as described in the
25 above paragraph, the process may be used to isolate only Factor IX.

Brief Description of the Drawings

Figure 1 shows a process in accordance with the invention, showing additional purification of the isolated protein fractions.

30 Figure 2 shows product recovery using a process in accordance with one embodiment of the invention.

Figure 3 shows an SDS-PAGE analysis in accordance with one embodiment of the first aspect of the invention.

35 Figure 4 shows a Single Radial Immunodiffusion analysis on fractions obtained from one embodiment of the first aspect of the invention.

Figure 5 illustrates one embodiment of the invention wherein the first and second aspects of the invention are used in series to isolate certain protein fractions.

Figure 6 shows an SDS-PAGE analysis of fractions from Factor VIII adsorption. Lane 1 depicts raw plasma, lane 2 depicts the run-through fraction and lane 3 depicts elution of Factor VIII/Factor IX. The Factor VIII/Factor IX eluate was diluted relative to the elution volume for direct comparison with the applied plasma, i.e. yields may be visually estimated.

Figure 7 shows an SDS-PAGE analysis of protein fractions obtained from the protein isolation process of the first aspect in which the raw material (solution comprising said proteins) used is the run-through fraction obtained from the process of the second aspect. Lane 1 depicts human plasma, lane 2 depicts the run-through fraction (α -1-PI), lane 3 depicts elution 2 (Albumin), lane 4: depicts elution 3 (IgG) and lane 5 depicts elution 4 (Fibrinogen).

Figure 8 shows a single Radial Immunodiffusion analysis of the fractions obtained from the protein isolation process of the first aspect in which the raw material (solution comprising said proteins) used is the run-through fraction obtained from the process of the second aspect. Fractions 1 to 4 represent run-through fraction, elution 2, elution 3 and elution 4 respectively. Figure 8A shows quantitation of albumin, and Figure 8B shows quantitation of IgG. The upper two rows of 8A and 8B depict a standard curve of raw plasma 100-20 % (double determinations). The lower two rows of A and B: depict the following: 1: Run-through fraction; 2: Elution 2; 3: Elution 3; 4: Elution 4.

Definitions

The following are some definitions that may be helpful in understanding the description of the present invention.

In the context of this specification, the term "comprising" means "including principally, but not necessarily solely". Furthermore, variations of the word "comprising", such as "comprise" and "comprises", have correspondingly varied meanings.

In the context of the present invention, the terms "elution step", "elution" or "eluting" may be used interchangeably and are intended to refer to a step of obtaining a protein fraction comprising one or more proteins which may, or may not have been bound and subsequently released from the solid separation medium.

In the context of the present specification, the term "washing step" is intended to refer to a step of flushing the solid separation medium with a fluid which does not substantially release any proteins from the solid separation medium.

In the context of the present specification, the term "equilibration step" is intended
5 to refer to a step wherein sufficient solution is allowed to pass through the solid separation medium so that counter-ion concentration, conductivity and pH of the outgoing solution is about the same as that of the incoming solution.

In the context of the present specification the term "recombinant broth" refers to soluble proteins which have been expressed *in vitro* by genetically manipulated cells.
10 The proteins which may be expressed by these manipulated cells within the recombinant broth may include: coagulation pathway proteins eg Factor VII, Factor VIII, Factor IX or Factor XIII, immunoglobulins for example, IgG, IgA, IgM, IgD, or IgE, transferrin (Tf), fibrinogen or a derivative thereof, plasma protease inhibitor such as an antithrombin, e.g., antithrombin III, α 1-proteinase inhibitor, blood pro-coagulation protein, blood anti-
15 coagulation protein, cytokine, growth factor, albumin or a derivative thereof, thrombolytic agent, anti-angiogenic protein, insulin or a derivative thereof, α -1-proteinase inhibitor or a derivative thereof, such as α -1-antitrypsin, α -2-antiplasmin or a derivative thereof, C-1 esterase inhibitor, apolipoprotein, HDL, Fibronectin or a derivative thereof, beta-2-glycoprotein I, plasminogen, plasmin, plasminogen activator, plasminogen
20 inhibitor, urokinase or derivative thereof, streptokinase or a derivative thereof, inter- α -trypsin inhibitor, α -2-macroglobulin, amyloid protein, orosomucoid, ferritin, pre-albumin, GC-globulin, haemopexin and C3-complement.

In the context of the present specification, the term "blood plasma" refers to the liquid portion of the blood and is a complex solution comprising more than 90 percent
25 water. The major solute of plasma is a heterogeneous group of proteins. Other plasma constituents include fatty substances (lipids), inorganic electrolytes, glucose, amino acids, vitamins, hormones, and waste products of metabolism.

In the context of the specification, the term "blood serum" refers to blood plasma from which fibrinogen has been removed in the process of clotting.

30 In the context of the specification, the terms "cryosupernatant" and "cryoprecipitate" should be understood according to the following: Cryosupernatant is that solution produced from plasma following the removal of the cryoprecipitate. Cryoprecipitate is composed of those proteins precipitated from plasma by exposure of plasma to temperatures of between 1° C and 10° C.

In the context of the specification, the term "fractionated human plasma" should be understood as referring to any component or mixture of components of plasma that is derived from plasma being subjected to a separation process e.g., precipitation, filtration, chromatography etc.

5 In the context of the present specification, the term "run-through" should be understood as referring to a protein fraction obtained from the second aspect of the invention comprising the eluate obtained when the plasma solution is loaded onto the column, which has been mixed with the eluate obtained from the first elution step.

10 Detailed Description of the Invention

Protein Isolation

The first aspect of the invention relates to processes for isolating proteins from biological solutions. More particularly, the invention relates to processes for isolating human proteins from human blood plasma, cryosupernatant derived from human plasma, fractionated human plasma, cryoprecipitate derived from human plasma, from blood serum, or from recombinant broths.

The solution comprising the proteins may be diluted with another suitable fluid prior to contacting said solid separation medium. For example, the plasma may be diluted with demineralised water or an aqueous solution of one or more inorganic salts of strong mineral acids compatible with the proteins to be isolated, for example salts of hydrochloric acid, sulfuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulfate, potassium sulfate, ammonium sulfate.

The solution comprising the proteins may be diluted with another suitable fluid in a ratio of from about 1000:1 to about 1:1000. For example, the dilution ratio may be about 1000:1, about 750:1, about 500:1, about 250:1, about 100:1, about 50:1, about 25:1, about 10:1, about 7.5:1, about 5:1, about 3:1, about 2.5:1, about 2:1, about 1:1, about 1:2, about 1:2.5, about 1:3, about 1:5, about 1:7.5, about 1:10, about 1:25, about 1:50, about 1:100, about 1:250, about 1:500, about 1:750, or about 1:1000.

The pH of the solution comprising the proteins may be adjusted prior to contacting the fluid with the solid separation medium. Alternatively, the pH may be adjusted after the solution comprising the proteins has been contacted with the solid separation medium. The pH may be raised or the pH may be lowered. Alternatively, the pH may remain unchanged. The pH may be adjusted to a pH in the range from about 3.0 to about 6.0, or alternatively, the pH may be adjusted to a pH in the range from about 4.5 to about 6.0.

For example, the pH may be adjusted to a pH of about 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0. The pH may be adjusted using a suitable acid, e.g, hydrochloric acid, sulfuric acid, phosphoric acid, citric acid, succinic acid, acetic acid, etc.

The pH may be adjusted using a suitable base, for example, carbonate, bicarbonate, ammonia, hydroxide, etc. The pH may be adjusted with a suitable buffer system. Buffer systems are well known to those skilled in the art and include, for example, citrate, acetate, phosphate, formate, succinate, MES, ADA, bis-tris propane, PIPES, ACES, Imidazole, MOPS, TES, HEPES, HEPPS, TRICINE, Glycine Amide hydrochloride, TRIS, BICINE, Glycylglycine, Boric Acid, CHES, CAPS. Many buffer systems are commercially available, and may be obtained, for example from Sigma Chemical Company. Those skilled in the art would be able to identify suitable buffer systems in terms of the desired pH.

The eluants may differ in pH. The elution steps may comprise treating the solid separation medium with eluants of increasing pH, or decreasing pH.

The eluants may have approximately the same pH but may differ in ionic strength. For example, respective eluants may comprise different buffer systems, and/or optionally comprise additional salts, for example salts of hydrochloric acid, sulphuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulphate, potassium sulphate, ammonium sulphate.

The yield of each of the proteins eluted may be at least 50%, or at least 60 %, or at least 70%, or at least 80%, or at least 90% relative to the amount of protein present in the starting material.

The elution steps may be carried out at a temperature in the range of 0 °C to 40 °C. For example, the temperature may be about 5 °C, about 10 °C, about 15 °C, about 20 °C, about 25 °C, about 30 °C, about 35 °C, or about 40 °C.

The process of the first aspect may optionally include one or more washing steps. A washing step may be performed at any stage of the process of the first aspect, for example, prior to contacting the solid separation medium with the solution comprising the proteins, after contacting the solid separation medium with the solution comprising the proteins, or between each elution step. The washing step may be carried out using any solution that does not result in a loss of the biological function of the proteins, for example water, saline, or a buffer solution, for example citrate buffer.

A washing step may be carried out after eluting the solid separation medium with the second eluant. The washing buffer may comprise an inorganic or organic salt of relatively high lytrophobicity such as ammonium sulfate, sodium sulfate, potassium

sulfate, ammonium phosphate, sodium phosphate, potassium phosphate, ammonium citrate, sodium citrate, potassium citrate. The ionic strength of the washing buffer may be at least 0.001 S/cm, or alternatively at least 0.01 S/cm, or alternatively at least 0.1 S/cm or further, at least 1.0 S/cm.

5 The process of the first aspect may further include one or more equilibration steps. An equilibration step may be carried out prior to contacting the solid separation medium with said solution comprising the proteins. The equilibration step may comprise treating said solid separation medium with water or a suitable buffer solution to adjust the pH and ionic strength of the solid separation medium. The solution used for the equilibration step
10 may be demineralised water or an aqueous solution of one or more inorganic salts of strong mineral acids compatible with the proteins to be isolated, e.g. salts of hydrochloric acid, sulfuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulfate, potassium sulfate, ammonium sulfate. Alternatively, the equilibration buffer may be an aqueous buffer solution comprising a
15 salt of an inorganic and/or an organic acid compatible with the proteins to be isolated, for example a buffer comprising a citrate, an acetate, a succinate, a lactate, a tartrate, a formate, a propionate, a phosphate, or a borate.

Equilibration steps may be carried out at a temperature in the range of about 10 °C to about 40 °C. For example, the temperature may be about 10 °C, about 15 °C, about 20
20 °C, about 25 °C, about 30 °C, or about 40°C.

The pH of the solution comprising the proteins may be the same as the pH of the buffer solution used to equilibrate the solid separation medium prior to contacting said solid separation medium with said solution comprising the proteins. The pH of the solution comprising the proteins may be different from the pH of the buffer solution used
25 to equilibrate the solid separation medium prior to contacting said solid separation medium with said solution comprising the proteins.

The process of the first aspect may further include one or more regeneration steps. A regeneration step comprises treating the solid separation medium with a suitable reagent capable of removing residual material from the solid separation medium. A
30 regeneration step may be carried out at any time after the first elution step is performed. Suitable regeneration reagents include bases, for example, hydroxide solutions, such as sodium hydroxide or potassium hydroxide, solutions of peracids or hydrogen peroxide, solutions comprising active chlorine, such as hypochlorite solutions, denaturants, such as guanidinium hydrochloride, organic solvents, e.g. ethanol.

In accordance with the process of the first aspect, the solid separation media may be loaded into a suitable chromatography column apparatus. The process may be carried out in a packed bed column in packed bed mode. Alternatively, the process may be carried out in an expanded bed absorption (EBA) column in expanded bed mode. In the context of the first aspect, practically all human plasma proteins can be adsorbed to the solid separation medium in a single column. Successive elution steps described herein can then be used to selectively elute protein fractions enriched in specific proteins.

EBA columns are well known in the art and suitable column apparatus and set up, including methods of introducing liquids into an expanded bed column, are available commercially from GE Healthcare, Sweden, or have been described in WO 99/65586, WO 01/85329 and WO92/00799, the entire contents of which are incorporated herein by cross-reference.

One embodiment of the first aspect comprises selecting an EBA column and placing an adequate quantity of solid separation medium into the column. The amount of solid separation medium used will depend on the amount of solution comprising the proteins that is to be applied, and the protein concentration in the protein solution. When the protein solution is human plasma or cryoprecipitated plasma, typically 1.0 litre of solid separation medium is used for every 0.5 to 1.5 litres of plasma. If the protein solution is a recombinant fermentation broth, one litre of solid separation medium may be used for 1 litre of fermentation broth and up to 1000 l of fermentation broth. A flow through is established from the bottom of the column until the solid separation medium is fluidised. Suitable column linear flow rates include flow rates in the range 0.5 to 20 cm/min, or alternatively from about 5 cm/min to 15 cm/min. The solid separation medium may then be equilibrated using an appropriate solution (for example, water, an aqueous electrolyte solution, or buffer solution), after which a solution, such as blood plasma, blood serum, cryosupernatant, solubilised cryoprecipitate or recombinant broth may be introduced to the bottom of the column. A further washing step may optionally be performed once the solution has been loaded onto the solid separation medium. Elution steps are then carried out in order to selectively elute protein fractions enriched in specific proteins.

Each respective elution step is carried out under conditions suitable to selectively elute a protein fraction comprising one or more proteins. For example, by varying the pH and/or ionic strength of the eluant used in respective elution steps, different proteins may be eluted. The eluant may be adjusted to a suitable pH and ionic strength using an appropriate buffer. The ionic strength may also be adjusted using a salt. Successive elution steps may comprise treating the solid separation medium with an eluant of

increasing pH. Alternatively successive elution steps may comprise treating the solid separation medium with an eluant of decreasing pH.

Another embodiment in accordance with the first aspect involves establishing an EBA column comprising agarose-tungsten carbide conglomerate beads functionalised with 2-mercaptosuccinic acid. The agarose-tungsten carbide beads have a size distribution between 40-120 micron with a mean diameter of 70 micron. The density of the beads is 2.9 g/ml. An equilibration step is carried out using a citrate buffer at a pH of about 4.5, which is immediately followed by a further equilibration step with citrate buffer at a pH of about 5.0. Human plasma that has been diluted with 2 parts water, and adjusted to a pH of 5.0 with HCl was then applied to the column in a ratio 1.5 litres of diluted plasma per litre of solid separation medium. The column was then eluted with the following buffer solutions:

Elution 1. 10mM sodium citrate at pH 5.0

Elution 2. 5g / litre of sodium caprylate and HCl at pH 6.0

Elution 3. 1M sodium citrate at pH 8.0

Elution 4. 20mM sodium citrate and 0.1M sodium chloride at pH 8.0

The solid separation medium was then regenerated with 1M NaOH.

The flow rate for all operations was 7.5 cm/minute, and the amounts of each solution used are given below in Table 2.

The volume of eluant used will typically depend on a number of interrelated factors, for example:

(i) The flow rate used during sample application, washing, elution, regeneration and equilibration;

(ii) The number of product fractions eluted;

(iii) The choice of eluants used in each step, as the choice of eluants influence the yield and purity of the individual fractions;

(iv) The optimal gap between individual fractions, which also has an influence on the yield and purity of the products obtained;

(v) Bed height of the solid separation medium, as generally the washing and elution volumes consumed decrease when the bed height of the solid separation medium is decreased.

Table 2 shows the optimal solution volume for each step in the above described embodiment.

Referring to Figure 3, it can be seen from the above embodiment that the proteins α -1 proteinase inhibitor, albumin, IgG and fibrinogen can be effectively isolated.

TABLE 2

Step	Volume of Solution*
Equilibration	5.0 CV
Eluant 1	4.2 CV
Eluant 2	2.9 CV
Eluant 3	4.4 CV
Eluant 4	2.1 CV
Regeneration	1.0 CV
TOTAL	19.6 CV

*CV is the column volume

- 5 Table 3 below shows the yields of the proteins relative to the applied raw plasma as determined by SRI.

TABLE 3

	Elution 1	Elution 2	Elution 3	Elution 4
Albumin	< 5%	95+%		
Immunoglobulin			95+%	<5%
α -1-proteinase inhibitor	95+%			
Fibrinogen			<5%	95+%

- 10 Single radial immunodiffusion (SRI) was performed in order to determine the relative yield of the individual proteins in eluant fractions 1 to 4. Figure 4 shows the SRI analysis for albumin, IgG, α -1-proteinase inhibitor and fibrinogen.

- The above described process was found to run in a smooth, reproducible and uncomplicated manner. All final eluates were found to be clear liquids with no sign of
 15 significant denaturation/precipitation of any of the proteins. Post column testing by SDS-PAGE and SRI as described above showed no sign of break-down, agglomeration or changes in immunoreactivity of the eluted products.

- Those skilled in the art will be aware that the process of the first aspect, and indeed the second, aspect, may be monitored by measuring the ultraviolet absorbance of the
 20 liquid exiting the column. The presence of proteins and other UV-absorbing material can be detected and quantified during the processes of the invention and correct collection of

the different protein fractions can be performed. Also continuous monitoring of pH, conductivity and refractive index may be useful in documenting and controlling the processes of the invention.

5 Isolation of Factor VIII and/or Factor IX

The solution comprising said Factor VIII and/or Factor IX is typically raw undiluted plasma which may be directly contacted with the solid separation medium. Alternatively, the raw plasma may be diluted with another suitable fluid prior to contacting said solid separation medium. For example, the plasma may be diluted with demineralised water or
10 an aqueous solution of one or more inorganic salts of strong mineral acids compatible with Factor VIII and/or Factor IX, for example salts of hydrochloric acid, sulfuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulfate, potassium sulfate, ammonium sulfate.

The solution comprising the Factor VIII and/or Factor IX may be diluted with
15 another suitable fluid in a ratio of from about 1000:1 to about 1:1000. For example, the dilution ratio may be about 1000:1, about 750:1, about 500:1, about 250:1, about 100:1, about 50:1, about 25:1, about 10:1, about 7.5:1, about 5:1, about 3:1, about 2.5:1, about 2:1, about 1:1, about 1:2, about 1:2.5, about 1:3, about 1:5, about 1:7.5, about 1:10, about 1:25, about 1:50, about 1:100, about 1:250, about 1:500, about 1:750, or about 1:1000.

20 The pH of the solution comprising Factor VIII and/or Factor IX may be adjusted prior to contacting with the solid separation medium. Alternatively, the pH may be adjusted after the solution comprising the Factor VIII and/or Factor IX has been contacted with the solid separation medium. The pH may be raised or the pH may be lowered. Alternatively, the pH may remain unchanged. The pH may be adjusted to a pH in the
25 range from about 5.0 to about 9.0. For example, the pH may be adjusted to a pH of about 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 or 9.0. The pH may be adjusted using a suitable acid, e.g. hydrochloric acid, sulfuric acid, phosphoric acid, citric acid, succinic acid, acetic acid, etc.

The pH may be adjusted using a suitable base, for example, carbonate, bicarbonate,
30 ammonia, hydroxide, etc. The pH may be adjusted with a suitable buffer system. Buffer systems are well known to those skilled in the art and include, for example, citrate, acetate, phosphate, formate, succinate, MES, ADA, bis-tris propane, PIPES, ACES, Imidazole, MOPS, TES, HEPES, HEPPS, TRICINE, Glycine Amide hydrochloride, TRIS, BICINE, Glycylglycine, Boric Acid, CHES, CAPS. Many buffer systems are
35 commercially available, and may be obtained, for example from Sigma Chemical

Company. Those skilled in the art would be able to identify suitable buffer systems in terms of the desired pH.

The process may optionally include one or more washing steps. A washing step may be performed prior to contacting the solid separation medium with the solution comprising the Factor VIII and/or Factor IX, or alternatively after contacting the solid separation medium with the solution comprising the Factor VIII and/or Factor IX. The washing step may be carried out using any solution that does not result in a loss of the biological function of the Factor VIII and/or Factor IX, for example water, saline, or a buffer solution, for example citrate buffer.

The washing buffer may alternatively comprise an inorganic or organic salt of relatively high lytrophobicity such as ammonium sulfate, sodium sulfate, potassium sulfate, ammonium phosphate, sodium phosphate, potassium phosphate, ammonium citrate, sodium citrate, potassium citrate. The washing buffer may have a pH in the range of about 5.0 to about 9.0. The pH of the washing buffer may be 5.2, 5.4, 5.6, 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8 or 9.0. The washing buffer may have a conductivity in the range from about 0.1 mS/cm to about 100 mS/cm. Alternatively, the washing buffer may have a conductivity of about 0.1mS/cm to about 40 mS/cm.

The washing buffer may also comprise an aqueous solution of one or more inorganic salts of strong mineral acids that result in no loss of the biological function of the proteins, for example salts of hydrochloric acid, sulphuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulphate, potassium sulphate, ammonium sulphate.

The process may further include one or more equilibration steps. An equilibration step may be carried out prior to contacting the solid separation medium with the solution comprising Factor VIII and/or Factor IX. The equilibration step may comprise treating said solid separation medium with water or a suitable buffer solution to adjust the pH and ionic strength of the solid separation medium. The solution used for the equilibration step may be demineralised water or an aqueous solution of one or more inorganic salts of strong mineral acids compatible with Factor VIII and/or Factor IX, e.g. salts of hydrochloric acid, sulfuric acid and nitric acid, such as sodium chloride, potassium chloride, ammonium chloride, sodium sulfate, potassium sulfate, ammonium sulfate. Alternatively, the equilibration buffer may be an aqueous buffer solution comprising a salt of an inorganic and/or an organic acid compatible with Factor VIII and/or Factor IX,

for example a buffer comprising a citrate, an acetate, a succinate, a lactate, a tartrate, a formate, a propionate, a phosphate, or a borate.

Equilibration steps may be carried out at a temperature in the range of about 2 °C to about 28 °C. For example, the temperature may be about 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 or 28 °C.

The pH of the solution comprising the Factor VIII and/or Factor IX may be the same as the pH of the buffer solution used to equilibrate the solid separation medium prior to contacting said solid separation medium with said solution comprising the Factor VIII and/or Factor IX. The pH of the solution comprising the Factor VIII and/or Factor IX may be different from the pH of the buffer solution used to equilibrate the solid separation medium prior to contacting said solid separation medium with said solution comprising the Factor VIII and/or Factor IX.

The process may further include one or more regeneration steps. A regeneration step comprises treating the solid separation medium with a suitable reagent capable of removing residual material from the solid separation medium. A regeneration step may be carried out after eluting said solid separation medium with an eluant to elute Factor VIII and/or Factor IX. Suitable regeneration reagents include bases, for example, hydroxide solutions, such as sodium hydroxide or potassium hydroxide, solutions of peracids or hydrogen peroxide, solutions comprising active chlorine, such as hypochlorite solutions, denaturants, such as guanidinium hydrochloride, organic solvents, e.g, ethanol.

The process may be carried out in a packed bed column in packed bed mode. Alternatively, the process may be carried out in an EBA column in expanded bed mode.

In one embodiment, as indicated above, the process may be used to isolate Factor VIII and Factor IX as a mixture from a solution comprising Factor VIII and Factor IX as well as other substances. In this embodiment an EBA column is selected and into it is placed an adequate quantity of solid separation medium. The amount of solid separation medium used will depend on the amount of solution comprising the Factor VIII and Factor IX that is to be applied, and the concentration of the Factor VIII and Factor IX in the solution. When the protein solution is human plasma or cryoprecipitated plasma, typically 1.0 litre of solid separation medium is used for every 5 to 30 litres of plasma. If the protein solution is a recombinant fermentation broth, one litre of solid separation medium may be used for 1 litre of fermentation broth and up to 1000 l of fermentation broth. A flow through is established from the bottom of the column until the solid separation medium is fluidised. Suitable column linear flow rates include flow rates in the range 0.5 cm/min to 40 cm/min, or alternatively from about 3 cm/min to 15 cm/min.

An equilibration step may then be performed using an appropriate solution (for example, water, an aqueous electrolyte solution, or buffer solution), after which a solution comprising Factor VIII and Factor IX, for example crude blood plasma, may be introduced to the bottom of the column, whereby the solution comprising Factor VIII and Factor IX is contacted with the solid separation medium. A first elution step is then performed in order to elute non-bound protein from the column. A washing step may optionally be performed after the first elution step. A second elution step is then performed in order to elute the Factor VIII and Factor IX from the solid separation medium.

The first and second aspects of the invention can be used in series in a two-step process wherein the first step involves use of the second aspect of the invention for the isolation of Factor VIII, or its complex with von Willebrand factor, and/or Factor IX from a solution comprising any of the proteins listed above on page 4 of the specification, with an enrichment factor of at least 10, and the second step involves use of the first aspect of the invention for the separation of the non-bound proteins obtained from the first step of the second aspect, into fractions comprising proteins such as α -1-proteinase inhibitor, albumin, IgG, anti-thrombin III and fibrinogen, all in high yield.

The process of the second aspect may be used in series with other protein isolation processes. For example, the process of the second aspect may be used to isolate Factor VIII and/or Factor IX from a solution of blood plasma, with the remaining proteins present in the fraction obtained from the run-through being used as the raw material from which further proteins, for example albumin, fibrinogen, immunoglobulins, α -1-proteinase inhibitor, may be isolated using various other fractionation processes eg precipitation; filtration, ion exchange chromatography etc.

Isolated proteins of the first and second aspects of the invention aspects may be incorporated into formulations as appropriate, for examples, as powders, solutions, liquids etc. Examples of some product formulations are provided in Table 4.

Table 4

Product	Formulation
Factor VIII	Dried Powder: 250 IU, 500 IU, 1000 IU
α -1-PI	Dried Powder, 1g
Albumin	5%, 25% solution
Antithrombin III	Dried Powder 1000 IU
IVIG	10% liquid, pH 4.25
Fibrinogen	Dried powder 1.5 g

Additional purification of the isolated protein fractions may also be employed in the processes of the invention as required, or as appropriate.

One such method may involve the serial inclusion of anion exchange or hydrophobic interaction chromatography steps. Methods such as these function to concentrate and further purify the protein fractions obtained from the processes of the invention. By choosing optimal stationary media and elution conditions for the serial chromatography step or steps, all of the proteins obtained from the processes of the invention could be bound onto a column. Bound protein could then be selectively eluted from the secondary column under a different set of chemical conditions resulting in significant concentration and potentially further purification of the proteins. Other chromatographic techniques, such as affinity chromatography, metal chelate chromatography and gel filtration may also be employed either individually, or in combination.

Figure 1 demonstrates some of the additional purification steps that may feasibly be employed following the processes of the invention.

The processes of the invention may optionally include viral inactivation steps. For example, viral inactivation/elimination steps are illustrated in Table 5 for each product, wherein each product has been isolated via a process of the invention.

Table 5

Product	Step 1	Step 2
Factor VIII	Solvent detergent	Dry heat, 80°C, 72h
α -1 proteinase inhibitor	Solvent detergent	Viral filtration
Antithrombin III	Pasteurization, 60°C, 1h	Viral filtration
Albumin	S-200 chromatography	Pasteurisation, 60°C, 1h
IVIG	Solvent detergent	Viral filtration
Fibrinogen	Solvent detergent	Wet heat, 62°C, 10h

The invention will now be described in more detail by way of illustration only, with respect to the following examples. The examples are intended to serve to illustrate the invention and should not be construed as limiting the generality of the disclosure of the description throughout the specification.

Examples

Example 1 - Isolation of α -1-proteinase inhibitor, antithrombin III, fibrinogen, immunoglobulins and albumin

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Step 1. Solid separation medium: Agarose – tungsten carbide beads functionalised with 2-mercaptosuccinic acid. The agarose-tungsten carbide beads have a size distribution of between 40-120 micron with a mean diameter of 70 micron. The density of the beads were 2.9 g/ml (FastLine UFC NNSDW cat. No.: CS48, UpFront Chromatography A/S, Copenhagen, Denmark.). The beads are placed in an EBA column (FastLine 100, UpFront Chromatography A/S, Copenhagen, Denmark) (10 cm diameter; 50 cm settled bed height; Settled bed Volume = 3.926 L). Equilibration is performed at a temperature of 25°C with 2.5 column volumes (9.8L) of 40 mM sodium citrate with a pH 4.5, followed by a further 2.5 column volumes (9.8L) of 40 mM sodium citrate and pH 5.0 at a linear flow rate of 7.5 cm/min.

10

Step 2. Onto this column was loaded 6L of a diluted plasma solution comprising 2L of thawed human plasma which had been diluted at a ratio of 1:2 with 4L of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of diluted plasma solution per litre of resin.

20

Step 3. Following loading of the diluted plasma solution, the column was eluted with Elution Buffer 1 comprising 4.2 column volumes (16.49L) of a buffer solution comprising 10mM sodium citrate pH 5.0. This resulted in the removal of non-bound protein, lipid and other substances including greater than 95% of the α -1-proteinase Inhibitor present in the diluted plasma solution applied to the column.

25

Step 4. Following Step 3 the column was eluted with 2.9 column volumes (11.39L) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of albumin from the column at a yield of greater than 95% of the quantity of the albumin present in the diluted plasma solution applied to the column. This step also results in the elution of 60% of the Antithrombin III present in the diluted plasma solution applied to the column.

30

Step 5. Following the elution of the column as described in Step 4 above the column was eluted with 4.4 column volumes (17.27L) of Elution Buffer 3, comprising 1M sodium citrate pH 8.0. This step resulted in the elution of greater than 95% of the immunoglobulins present in the diluted plasma solution applied to the column.

35

Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising 2.1 column volumes (8.24L) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 8.0. This Step resulted in the elution of greater than 95% of the fibrinogen present in the diluted plasma solution applied to the column.

Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (3.926L) of 1M sodium hydroxide and re-equilibrated with 2.5 column volumes (9.8L) of 40 mM sodium citrate pH 4.5 followed by a further 2.5 column volumes (9.8L) of 40 mM sodium citrate and pH 5.0.

Example 2 - Isolation of α -1-proteinase inhibitor, albumin, immunoglobulins and fibrinogen wherein an extra washing step is included

The solid separation medium used in this Example is the same as that used in Example 1.

Step 1. An EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (196.3mL) of 40 mM sodium citrate pH 4.5 at a linear flow rate of 5.0 cm/min.

Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

Step 3. Following loading of the diluted plasma solution, the column was eluted with 3.3 column volumes (259.2mL) of Elution Buffer 1 comprising 10mM sodium citrate pH 5.0. This resulted in the removal of non-bound protein, lipid and other substances including the α -1-proteinase Inhibitor present in the diluted plasma solution applied to the column.

Step 4. Following Step 3 above, the column was eluted with 2.6 column volumes (204.2mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of the albumin present in the diluted plasma solution applied to the column.

Step 4a. Following elution of the column as described in Step 4 above the column was washed with 1.0 column volume (78.5mL) of 1M sodium citrate pH 8.0.

Step 5. Following the washing of the column as described in Step 4a above the column was eluted with 4.9 column volumes (384.8mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 8.0. This step resulted in the elution of the immunoglobulins present in the diluted plasma solution applied to the column.

5 Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 2.6 column volumes (204.2mL) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of the fibrinogen present in the diluted plasma solution applied to the column.

10 Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (78.5mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (157mL) of 40 mM sodium citrate pH 4.5.

Example 3 - Isolation of α -1-proteinase inhibitor, albumin, transferrin, immunoglobulins and fibrinogen

The solid separation medium used in this Example is the same as that used in Example 1.

20 Step 1. An EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (196.3mL) of 40 mM sodium citrate pH 5.0 at a linear flow rate of 15.0 cm/min.

25 Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

30 Step 3. Following loading of the diluted plasma solution, the column was eluted with Elution Buffer 1 comprising 9.4 column volumes (738.3mL) of demineralised water. This resulted in the removal of non-bound protein, lipid and other substances including 100% of the α -1-proteinase Inhibitor, 10% of the Albumin, 5% of the Transferrin and 10% of the Fibrinogen present in the diluted plasma solution applied to the column.

35 Step 4. Following Step 3 above the column was eluted with 8.9 column volumes (699mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of albumin from the column at a yield of 90% of the quantity of the

albumin present in the diluted plasma solution applied to the column. This step also results in the elution of 5% of the immunoglobulins present in the diluted plasma solution applied to the column.

Step 5. Following the eluting of the column as described in Step 4 above the column was eluted with 9.0 column volumes (706.8 mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 8.0. This step resulted in the elution of greater than 85% of the immunoglobulins present in the diluted plasma solution applied to the column. This step also results in the elution of 95% of the transferrin and 30% of the fibrinogen present in the diluted plasma solution applied to the column.

Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 5.0 column volumes (392.7mL) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of 60% of the fibrinogen and 10% of the immunoglobulins present in the diluted plasma solution applied to the column.

Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (78.5mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (157mL) of 40 mM sodium citrate pH 5.0.

Example 4 - Isolation of α -1-proteinase inhibitor, albumin, immunoglobulins, transferrin and fibrinogen

The solid separation medium used in this Example is the same as that used in Example 1.

Step 1. An EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (196.3mL) of 40 mM sodium citrate pH 5.0 at a linear flow rate of 5.0 cm/min.

Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

Step 3. Following loading of the diluted plasma solution, the column was eluted with Elution Buffer 1 comprising 6.8 column volumes (533.8mL) of demineralized water. This resulted in the removal of non-bound protein, lipid and other substances including

100% of the α -1-Proteinase Inhibitor, 10% of the Albumin, 5% of the Transferrin and 10% of the Fibrinogen present in the diluted plasma solution applied to the column.

Step 4. Following washing of the column as described in Step 3 above the column was eluted with 5.5 column volumes (431.75mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of albumin from the column at a yield of 90% of the quantity of the albumin present in the diluted plasma solution applied to the column. This step also results in the elution of 5% of the immunoglobulins present in the diluted plasma solution applied to the column.

Step 5. Following Step 4 above the column was eluted with 5.0 column volumes (392.5mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 8.0. This step resulted in the elution of greater than 85% of the immunoglobulins present in the diluted plasma solution applied to the column. This step also results in the elution of 95% of the transferrin and 30% of the fibrinogen present in the diluted plasma solution applied to the column.

Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 3.1 column volumes (243.35mL) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of 60% of the fibrinogen and 10% of the immunoglobulins present in the diluted plasma solution applied to the column.

Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (78.5mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (157mL) of 40 mM sodium citrate pH 5.0.

Example 5 - Isolation of α -1-proteinase inhibitor, albumin, immunoglobulins, fibrinogen and transferrin

The solid separation medium used in this Example is the same as that used in Example 1.

Step 1. An EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (196.3mL) of 40 mM sodium citrate pH 5.0 at a linear flow rate of 10.0 cm/min.

Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior

to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

Step 3. Following loading of the diluted plasma solution, the column was eluted with Elution Buffer 1 comprising 9.5 column volumes (745.75mL) of demineralized water.

- 5 This resulted in the removal of non-bound protein, lipid and other substances including 100% of the α -1-Proteinase Inhibitor, 10% of the Albumin, 5% of the Transferrin and 10% of the Fibrinogen present in the diluted plasma solution applied to the column.

- Step 4. Following Step 3 above the column was eluted with 7.1 column volumes (557.35mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4
10 resulted in the elution of albumin from the column at a yield of 90% of the quantity of the albumin present in the diluted plasma solution applied to the column. This step also results in the elution of 5% of the immunoglobulins present in the diluted plasma solution applied to the column.

- Step 5. Following Step 4 above the column was eluted with 5.9 column volumes
15 (463.15mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 8.0. This step resulted in the elution of greater than 85% of the immunoglobulins present in the diluted plasma solution applied to the column. This step also results in the elution of 95% of the transferrin and 30% of the fibrinogen present in the diluted plasma solution applied to the column.

- 20 Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 3.1 column volumes (243.35mL) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of 60% of the fibrinogen and 10% of the immunoglobulins present in the diluted plasma solution applied to the column.

- 25 Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (78.5mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (157mL) of 40 mM sodium citrate pH 5.0.

Example 6 - Isolation of α -1-proteinase inhibitor, albumin, immunoglobulins, transferrin and fibrinogen

5 The solid separation medium used in this Example is the same as that used in Example 1.

Step 1. An EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (196.3mL) of 40 mM sodium citrate pH 5.0 at a linear flow rate of 20.0 cm/min.

10 Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

15 Step 3. Following loading of the diluted plasma solution, the column was eluted with Elution Buffer 1 comprising 12.6 column volumes (989.1mL) of demineralized water. This resulted in the removal of non-bound protein, lipid and other substances including 100% of the α -1-Proteinase Inhibitor present in the diluted plasma solution applied to the column. It is noted that a significant amount of albumin is being also eluted in this step at a flow rate of 20 cm/min.

20 Step 4. Following Step 3 above the column was eluted with 10.6 column volumes (832.1mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of albumin from the column at a yield of 90% of the quantity of the albumin present in the diluted plasma solution applied to the column. This step also results in the elution of 5% of the immunoglobulins present in the diluted plasma solution applied to the column.

25 Step 5. Following Step 4 above the column was eluted with 6.9 column volumes (541.65mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 8.0. This step resulted in the elution of greater than 85% of the immunoglobulins present in the diluted plasma solution applied to the column. This step also results in the elution of 95% of the transferrin and 30% of the fibrinogen present in the diluted plasma solution applied to the column.

30 Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 6.8 column volumes (533.8mL) of 20mM

sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of 60% of the fibrinogen and 10% of the immunoglobulins present in the diluted plasma solution applied to the column.

5 Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (78.5mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (157mL) of 40 mM sodium citrate pH 5.0.

Example 7 - Isolation of α -1-proteinase inhibitor, albumin and IgG

10 The solid separation medium used in this Example is the same as that used in Example 1.

Step 1. An EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (196.3mL) of 40 mM sodium citrate pH 4.5 at a linear flow rate of 5.0 cm/min.

15 Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

Step 3. Following loading of the diluted plasma solution, the column was eluted with 3.3 column volumes (259.2mL) of Elution Buffer 1 comprising 10mM sodium citrate pH 5.0. This resulted in the removal of non-bound protein, lipid and other substances including the α -1-Proteinase Inhibitor present in the diluted plasma solution applied to the column.

25 Step 4. Following washing of the column as described in Step 3 above, the column was eluted with 2.7 column volumes (211.95mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of the albumin present in the diluted plasma solution applied to the column.

Step 5. Deleted.

30 Step 6. Following the elution of the column as described in Step 4 above, the column was eluted with Elution Buffer 4, comprising of 3.8 column volumes (298.3mL) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of the IgG present in the diluted plasma solution applied to the column.

Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (78.5mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (157mL) of 40 mM sodium citrate pH 4.5.

5 **Example 8- Isolation of α -1-proteinase inhibitor, albumin, immunoglobulins and fibrinogen wherein an extra washing step is included**

The solid separation medium used in this Example is the same as that used in Example 1.

10 Step 1 EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (392.5mL) of 40 mM sodium citrate pH 4.5 at a linear flow rate of 5.0 cm/min.

15 Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

20 Step 3. Following loading of the diluted plasma solution, the column was eluted with 2.9 column volumes (455.3mL) of Elution Buffer 1 comprising 10mM sodium citrate pH 5.0. This resulted in the removal of non-bound protein, lipid and other substances including the α -1-Proteinase Inhibitor present in the diluted plasma solution applied to the column.

25 Step 4. Following Step 3 above, the column was eluted with 2.8 column volumes (439.6mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of the albumin present in the diluted plasma solution applied to the column.

Step 4a. Following elution of the column as described in Step 4 above the column was washed with 1.0 column volume (157mL) of 1M sodium citrate pH 8.0.

30 Step 5. Following the washing of the column as described in Step 4a above the column was eluted with 4.5 column volumes (706.5mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 8.0. This step resulted in the elution of the immunoglobulins present in the diluted plasma solution applied to the column.

Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 1.9 column volumes (298.3mL) of 20mM

sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of the fibrinogen present in the diluted plasma solution applied to the column.

Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (157mL) of 1M sodium hydroxide and re-equilibrated with 1.9 column volumes (298.3mL) of 40 mM sodium citrate pH 4.5.

Example 9 - Isolation of α -1-proteinase inhibitor, albumin, immunoglobulins and fibrinogen wherein an extra washing step is included

The solid separation medium used in this Example is the same as that used in Example 1.

Step 1. EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen Denmark) (2 cm diameter; 25 cm bed height; Settled bed Volume = 78.5mL), was equilibrated at a temperature of 21°C with 2.5 column volumes (392.5mL) of 40 mM sodium citrate pH 4.5 at a linear flow rate of 10.0 cm/min.

Step 2. Onto this column was loaded 117.8mL of a diluted plasma solution comprising 39.3mL of thawed human plasma which had been diluted at a ratio of 1:2 with 78.5mL of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

Step 3. Following loading of the diluted plasma solution, the column was eluted with 3.7 column volumes (580.9mL) of Elution Buffer 1 comprising 10mM sodium citrate pH 5.0. This resulted in the removal of non-bound protein, lipid and other substances including the α -1-Proteinase Inhibitor present in the diluted plasma solution applied to the column.

Step 4. Following Step 3 above, the column was eluted with 3.6 column volumes (565.2mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of the albumin present in the diluted plasma solution applied to the column.

Step 4a. Following elution of the column as described in Step 4 above the column was washed with 1.0 column volume (157mL) of 1M sodium citrate pH 8.0.

Step 5. Following the washing of the column as described in Step 4a above the column was eluted with 5.4 column volumes (847.8mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 8.0. This step resulted in the elution of the immunoglobulins present in the diluted plasma solution applied to the column.

Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 1.7 column volumes (266.9mL) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 8.0. This step resulted in the elution of the fibrinogen present in the diluted plasma solution applied to the column.

- 5 Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (157mL) of 1M sodium hydroxide and re-equilibrated with 2.6 column volumes (408.2mL) of 40 mM sodium citrate pH 4.5.

Analytical Determinations:

- 10 All yields for the above examples were determined by comparative single radial immunodiffusion (Agnete Ingild in: Handbook of Immunoprecipitation-in-Gel Techniques, ed. Nils H. Axelsen, Scandinavian Journal of Immunology, Suppl. No. 10, Vol 17, pp.41-57, 1983.

Example 10 – Isolation of Factor VIII and Factor IX from raw plasma

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- Step 1. Solid separation medium: The matrix backbone is an agarose-tungsten carbide resin, the spacer is derived from an epoxy group and the ligand is *p*-xylylene-diamine or *m*-xylylene-diamine. The beads are placed in an EBA column (FastLine 10, UpFront Chromatography A/S, Copenhagen, Denmark) (1 cm diameter; 25cm settled bed height; Settled bed Volume = 20mL). Equilibration is performed at a temperature of 25°C 20 mM sodium citrate and pH 6.0 at a linear flow rate of 5.0 cm/min.

Step 2 (Loading step). Onto this column was loaded 300mL of undiluted raw plasma. The pH of the plasma was adjusted to pH 6.0 with 1M HCl prior to application to the column and the load ratio was 15L of plasma per litre of resin.

- 25 Step 3 (Elution 1). Following loading of the plasma, the column was eluted with 9 column volumes (180 mL) of Elution Buffer 1 comprising 20mM sodium citrate, pH 6.0. This resulted in the removal of non-bound protein.

Step 4. The column was then washed with 6.4 column volumes (128mL) of washing buffer, comprising of 20 mM sodium citrate + 0.2M sodium chloride, pH 6.0.

- 30 Step 5 (Elution 2). Following Step 4 above the column was eluted with 6.0 column volumes (120mL) of Elution Buffer 2, comprising 20mM sodium citrate + 1.0M sodium chloride, pH 8.0. This step resulted in the elution of greater than 45% of the Factor VIII activity present in plasma applied to the column. This step also results in the elution of 85% of the Factor IX activity present in the plasma applied to the column.

Step 6. Following elution of the column as described in Step 5 the column was regenerated with 1 column volume (20mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (40mL) of 20 mM sodium citrate pH 6.0.

Determination of Factor VIII activity:

5 Relative Factor VIII activities were determined in the raw plasma and EBA protein fractions using a Factor VIII activity kit from Coamatic (cat no 822585-63). A standard curve was produced using the raw plasma undiluted (100 % activity) and diluted to 80 %, 60 %, 40 % 20 % and 0 % (blank) of the initial activity (not shown). Fractions from run-through, wash and eluate (Elution 2) from the column were analysed and the activity yield
10 in the eluate was determined by reference to the standard curve (see table 7). From table 7 it can be seen that the solid separation medium binds and elutes 45 % of the Factor VIII activity under the conditions applied.

TABLE 7: Factor VIII, fraction volumes and activity yield

15

Volume	Relative Factor VIII activity		Activity yield
Raw plasma	300 ml	100 %	100 %
Run-through	480 ml	13 %	21 %
Wash	128 ml	0 %	0 %
Eluate (Elution 2)	120 ml	113 %	45 %

Determination of Factor IX antigen:

Relative Factor IX antigen concentrations were determined in the raw plasma and EBA protein fractions by a Factor IX sandwich ELISA using commercial antibodies. A
20 standard curve was produced using the raw plasma undiluted (100 %) and diluted to 80 %, 60 %, 40 % 20 % and 0 % (blank) of the initial concentration (not shown). Fractions from run-through, wash and eluate (Elution 2) from the column were analysed and the antigen yield in the eluate (Elution 2) was determined by reference to the standard curve (see Table 8). From table 8, it can be seen that the solid separation medium binds and
25 elutes 85 % of the Factor IX antigen under the conditions applied.

TABLE 8: Factor IX, fraction volumes and antigen yield

Volume	Relative Factor IX antigen concentration		Yield
Raw plasma	300 ml	100 %	100 %
Run-through	480 ml	0 %	0 %
Wash	128 ml	0 %	0 %
Eluate (Elution 2)	120 ml	213 %	85 %

Selectivity

Figure 8 illustrates by SDS-PAGE that the eluate (Elution 2) comprise a very small proportion of the applied protein. When diluted for direct comparison to the raw plasma only very faint bands of IgG, albumin and other proteins are visible indicating that the loss of these proteins to the FVIII/FIX protein fraction will be insignificant. Also the run-through fraction is diluted for direct comparison with the raw plasma and no significant loss of protein is observed. RID showed full recovery of α -1-P1 and fibrinogen in the run-through fractions as well (not shown).

Example 11 – Compatibility of the process of the second aspect with the process of the first aspect

The run-through fraction from Example 10 was used as a raw material in the process of the first aspect in order to determine whether the prior removal of Factor VIII and Factor IX exerted any negative influence on the isolation process of the first aspect.

Step 1. Solid separation medium: Agarose – tungsten carbide beads functionalised with 2-mercaptosuccinic acid. The agarose-tungsten carbide beads have a size distribution of between 40-120 micron with a mean diameter of 70 micron. The density of the beads were 2.9 g/ml (FastLine UFC NNSDW cat. No.: CS48, UpFront Chromatography A/S, Copenhagen, Denmark.). The beads are placed in an EBA column (FastLine 20, UpFront Chromatography A/S, Copenhagen, Denmark) (2cm diameter; 50 cm settled bed height; Settled bed Volume = 157mL). Equilibration is performed at a temperature of 25°C with 2.5 column volumes (392.5mL) of 40 mM sodium citrate with a pH 4.5, followed by a further 2.5 column volumes (392.5mL) of 40 mM sodium citrate and pH 5.0 at a linear flow rate of 7.5 cm/min.

Step 2. Onto this column was loaded the run-through from the column described in Example 10 adjusted to 1 part of raw plasma + 2 parts of water. The pH of the diluted plasma solution was adjusted to pH 5.0 with 1M HCl prior to application to the column and the load ratio was 1.5 litres of plasma solution per litre of resin.

5 Step 3. Following loading of the diluted plasma solution, the column was eluted with 3.3 column volumes (518mL) of Elution Buffer 1 comprising 10mM sodium citrate pH 5.0. This resulted in the removal of non-bound protein, lipid and other substances including the α -1-proteinase Inhibitor present in the diluted plasma solution applied to the column.

10 Step 4. Following Step 3 above, the column was eluted with 2.6 column volumes (408mL) of Elution Buffer 2, comprising 5 g/L sodium caprylate/HCl, pH 6.0. Step 4 resulted in the elution of the albumin present in the diluted plasma solution applied to the column.

15 Step 4a. Following elution of the column as described in Step 4 above the column was washed with 1.0 column volume (157mL) of 1M sodium citrate pH 8.0.

Step 5. Following the washing of the column as described in Step 4a above the column was eluted with 4.9 column volumes (769mL) of Elution Buffer 3, comprising 0.3M sodium citrate pH 7.4. This step resulted in the elution of the immunoglobulins present in the diluted plasma solution applied to the column.

20 Step 6. Following the elution of the column as described in Step 5 above, the column was eluted with Elution Buffer 4, comprising of 2.6 column volumes (408mL) of 20mM sodium citrate comprising 0.1M sodium chloride at pH 7.4. This step resulted in the elution of the fibrinogen present in the diluted plasma solution applied to the column.

25 Step 7. Following elution of the column as described in Step 6 the column was regenerated with 1 column volume (157mL) of 1M sodium hydroxide and re-equilibrated with 2.0 column volumes (314mL) of 40 mM sodium citrate pH 4.5.

30 4. As can be seen from Figure 7, the qualitative pattern obtained with the Factor VIII/Factor IX depleted plasma is identical to the pattern obtained in Example 4. The process volumes obtained for each fraction were also equal to those described in Example

 Figure 8A-B illustrates the quantitative analysis of albumin and IgG in the different protein fractions, and as can be seen practically all of the albumin is recovered in elution 2, and practically all of the IgG is recovered in elution 3. Albumin and IgG are barely detectable in the other fractions.

α -1-proteinase inhibitor and fibrinogen were also found to behave in the same way as described in the process of the first aspect.

The results demonstrate that *p*-xylylenediamine and *m*-xylylenediamine are capable of selectively extracting Factor VIII and Factor IX from undiluted plasma in an efficient manner without significantly reducing the levels of other plasma proteins, which may be isolated in a subsequent process(es), such as the process of the first aspect. The Factor VIII/Factor IX isolation process is compatible with the albumin/IgG/Fibrinogen/ α -1-proteinase inhibitor isolation process of the first aspect.

CLAIMS

1. A process for isolating proteins from a solution comprising said proteins, said solution being selected from the group consisting of: crude blood plasma, blood serum, cryosupernatant derived from plasma, fractionated human plasma, cryoprecipitate derived from plasma and recombinant broths, said process comprising:

(i) providing a solid separation medium having the formula:

M-S-L

wherein M is a matrix backbone, S is an optional spacer arm, and L is a ligand which is mercaptonicotinic acid;

(ii) contacting said solid separation medium with said solution, such that at least one of said proteins becomes reversibly bound to said solid separation medium;

(iii) performing at least one elution step to selectively elute from the solid separation medium, at least one protein fraction.

2. The process according to claim 1, wherein the solution is crude blood plasma.

3. The process according to claim 1 or claim 2, wherein M is a high density resin.

4. The process according to any one of claims 1 to 3, wherein L is 2-mercaptonicotinic acid.

5. The process of any one of claims 1 to 4, where S is a compound comprising an epoxy group.

6. The process according to any one of claims 1 to 5, wherein the at least one protein fraction comprises at least one protein selected from the group consisting of: immunoglobulins such as, IgG, IgA, IgM, IgD, or IgE, transferrin, fibrinogen or a derivative thereof, plasma protease inhibitor such as an antithrombin, for example, antithrombin III, blood pro-coagulation protein, blood anti-coagulation protein, cytokine, growth factor, albumin or a derivative thereof, thrombolytic agent, anti-angiogenic protein, insulin or a derivative thereof, α -1-proteinase inhibitor or a derivative thereof, such as α -1-antitrypsin, α -2-antiplasmin or a derivative thereof, C-1 esterase inhibitor, apolipoprotein, HDL, Fibronectin or a derivative thereof, beta-2-glycoprotein I, plasminogen, plasmin, plasminogen activator, plasminogen inhibitor, urokinase or derivative thereof, streptokinase or a derivative thereof, inter- α -trypsin inhibitor, α -2-macroglobulin, amyloid protein, orosomucoid, ferritin, pre-albumin, GC-globulin, haemopexin and C3-complement.

7. The process according to claim 6, wherein the at least one protein fraction comprises at least one protein selected from the group consisting of: immunoglobulins, transferrin, fibrinogen or a derivative thereof, α -1-proteinase inhibitor and albumin, or a derivative thereof.

5 8. The process according to any one of claims 1 to 7, wherein the solution comprising said proteins has a pH of between about 3.0 and about 6.0.

9. The process according to claim 8, wherein the solution comprising said proteins has a pH of between about 4.5 and about 6.0.

10 10. The process according to any one of claims 1 to 9, wherein a first elution step comprises eluting the solid separation medium with a solution having a pH of between about 4 and about 8, and an ionic strength of between about 0.00005 S/cm to about 0.1 S/cm to elute a first protein fraction.

11. The process according to claim 10, wherein the first protein fraction comprises at least one protein selected from the group consisting of: α -1 proteinase inhibitor, albumin, orosomucoid, and pre-albumin.

12. The process according to claim 10 or claim 11, wherein a second elution step comprises eluting the solid separation medium with a solution having a pH of between about 5 and about 7, and an ionic strength of between about 0.0001 S/cm to about 0.1 S/cm to elute a second protein fraction.

20 13. The process according to claim 12 wherein the solution further comprises an aromatic, non-aromatic or heteroaromatic hydrophobic compound.

14. The process according to claim 13, wherein the non-aromatic hydrophobic compound is a salt of an alkyl carboxylic acid, a salt of a sulfonic acid or a negatively charged detergent.

25 15. The process according to any one of claims 12 to 14, wherein the second protein fraction comprises at least one protein selected from the group consisting of: albumin and immunoglobulins.

16. The process according to any one of claims 12 to 15, wherein a third elution step comprises eluting the solid separation medium with a solution having a pH of between about 5 and about 9, and an ionic strength of between about 0.001 S/cm to about 4 S/cm to elute a third protein fraction.

17. The process according to claim 16, wherein the third protein fraction comprises at least one protein selected from the group consisting of: immunoglobulins, transferrin and fibrinogen.

18. The process according to claim 16 or claim 17, wherein a fourth elution step comprises eluting the solid separation medium with a solution having a pH of between about 5 and about 9, and an ionic strength of between about 0.01 S/cm to about 2 S/cm to elute a fourth protein fraction.

5 19. The process according to claim 18, wherein the solution further comprises a salt of a mineral acid.

20. The process according to claim 18 or claim 19, wherein the fourth protein fraction comprises at least one protein selected from the group consisting of: transferrin, immunoglobulins, fibrinogen and α -2-macroglobulin.

10 21. The process of any one of claims 1 to 20, wherein said process is carried out in an expanded bed adsorption column in expanded bed mode.

22. The process of any one of claims 1 to 20, wherein said process is carried out in a packed bed column in packed bed mode.

15 23. A process for isolating Factor VIII and/or Factor IX from a solution comprising Factor VIII and/or Factor IX, said solution being selected from the group consisting of: crude blood plasma, blood serum, cryosupernatant derived from plasma, fractionated human plasma, cryoprecipitate derived from plasma and recombinant broths, said process comprising:

(i) providing a solid separation medium having the formula:

20

M-S-L

wherein M is a matrix backbone, S is an optional spacer arm and L is a ligand which is xylylenediamine;

25 (ii) contacting said solid separation medium with said solution such that at least Factor VIII and/or Factor IX become reversibly bound to said solid separation medium;

(iii) performing a first elution step to elute non-bound proteins from the solid separation medium;

30 (iv) performing a second elution step to elute Factor VIII and/or Factor IX from the solid separation medium.

24. The process according to claim 23, wherein the solution comprising Factor VIII and Factor IX is crude blood plasma.

25. The process according to any one of claim 23 or claim 24, wherein M is a high density resin.

26. The process according to any one of claims 23 to 25, wherein S is a compound comprising an epoxy group.

27. The process according to any one of claims 23 to 26, wherein a first elution step comprises eluting the solid separation medium with a solution having a pH of
5 between about 5.5 and about 6.5, and an ionic strength of between about 0.001 S/cm to about 0.02 S/cm to elute a first protein fraction.

28. The process according to claim 27 wherein a second elution step comprises eluting the solid separation medium with a solution having a pH of between about 7 and about 9, and an ionic strength of between about 0.03 S/cm to about 0.2 S/cm to elute a
10 second protein fraction.

29. The process according to claim 27 or claim 28, wherein the solution further comprises a salt of a mineral acid.