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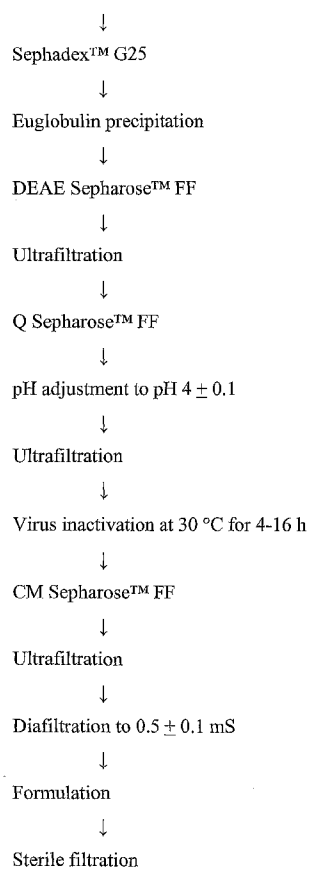
[Continued on next page]

(54) Title: A METHOD OF PRODUCING IGG

(57) Abstract: The present invention relates to a method for producing IgG from plasma, which method comprises buffering of fresh plasma; removal of euglobulins; removal of albumin; concentrating the IgG fraction so obtained; subjecting the plasma fraction to anion exchange wherein IgG is recovered in the flow-through; concentration of the IgG plasma fraction; carrying out virus inactivation in the IgG plasma fraction collected; removal of the chemicals added to inactivate virus by cation exchange; concentration of the IgG plasma fraction; formulation of the IgG plasma fraction; and sterile filtration.



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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

A METHOD OF PRODUCING IgG

Technical Field

The present invention relates to an improved method of producing IgG, preferably for medical applications. The invention also encompasses IgG isolated from plasma by a method according to the invention.

Background

IgG prepared from human plasma is widely used in the treatment of agammaglobulinaemia, idiopathic thrombocytopenic purpura and in the prophylaxis of certain diseases. IgG preparations are administered intramuscularly as well as intravenously.

Within the field of the art it is well known that isolated IgG preparations have marked anticomplementary activities (ACA). It has been shown that the components responsible for these activities are aggregates of IgG forming spontaneously or as a result of the isolation procedure. These anticomplementary aggregates have shown to be harmful in several clinical applications of the IgG products. For example, intravenous administration of IgG preparations can give rise to adverse side reactions, including anaphylactic shock.

Several solutions have been proposed to overcome the problem with ACA in IgG preparations. For example, in US 3 966 906 a process is described for treating a crude gamma globulin fraction of serum with pepsin to disaggregate IgG and thereby obtaining a low anticomplementary activity. However, the therapeutic effect provided by such a preparation is unacceptably short since it is rapidly excreted. Another drawback with the pepsin treated immunoglobulins is that their Fc binding capacity is lower than for native immunoglobulins.

Attempts have been made to stabilise pepsin treated IgG preparations, such as by polyethylene glycol, PEG, see for example WO 86/06993.

To solve the problem with high ACA activity proposals have also been made to chemically modify the IgG preparations. For example, in US 3 902 262 a portion of the disulphide linkages of the IgG molecule is reduced to –SH groups and then the –SH groups are alkylated.

For obvious reasons, it would be desirable to have an IgG product which is free from enzymatic and other chemical modification and to be as close to native as possible. A method fulfilling these criteria has been described in "An improved chromatographic method for production of IgG from human plasma" by I. Andersson, L-O Lindquist, J. Berglöf, presented at the "XXIII Congress of the ISBT", Amsterdam, The Netherlands, July 2-8, 1994). However, also this procedure shows unsatisfactory high ACA-levels and does not fulfil the current FDA and EU requirements for intravenous drugs.

US Pat. No. 6,835,379 (Lindquist) relates to a method of producing IgG products from human plasma. More specifically, a method is disclosed, which comprises at least:
(i') removal of albumin resulting in an IgG fraction,
(ii') purifying IgG from an IgG fraction, which is derived from the IgG fraction obtained in step (i'), by adsorbing IgG to a cation exchanger and collecting the adsorbed IgG fraction, and
(iii') virus inactivation in an IgG fraction derived from the IgG fraction collected in step (ii'),
wherein the IgG fraction obtained in step (i') is concentrated, the pH of the IgG fraction released from the cation exchanger used in step (ii') is adjusted to pH to 4±0.1, and the pH is maintained at below 6.0 during the remaining steps of the method. Further, the virus inactivation (step iii') may be carried out by using virus inactivation chemicals, preferably a solvent/detergent (S/D) solution, at a temperature of 30°C ± 2°C for at least 4 hours.

However, there is an ongoing need in this field of alternative methods, which speed up the purification process. As in many protein purification protocols, there is a general desire of reducing the number of process steps.

Brief description of the invention

A first aspect of the invention is to provide a method for producing IgG from plasma, which method is less time-consuming than the prior art methods. This can be achieved by a method as defined in the appended claims, which has been shown to give well comparable yields and quality of IgG as the prior art methods.

Another object is to provide a method for producing IgG from plasma, which uses less purification materials than the prior art methods.

A further object of the invention is to provide a method as discussed above, which results in an IgG preparation wherein the anticomplementary activity (ACA) is maintained at levels acceptable for medical purposes.

Other aspects and advantages will appear from the detailed description that follows.

Brief description of the drawing

Figure 1 shows schematically an illustrative embodiment of the method for producing IgG according to the invention.

Definitions

Anticomplementary activity (ACA) refers to measurements in the final product, and is measured according to Eur. Pharmacopoeia Monograph (1997) page 963 (2.6.17). ACA should not be higher than 1 CH_{50}/mg of immunoglobulin G.

Detailed description of the invention

In a first aspect, the present invention relates to a method for producing IgG from plasma, which method comprises:

a) buffering of fresh plasma;

b) removal of euglobulins;

c) removal of albumin followed by concentrating the IgG fraction so obtained;

d) subjecting the plasma fraction obtained from step (c) to anion exchange and collecting the unbound plasma fraction comprising IgG,

e) concentration of the IgG plasma fraction collected in step (d);

f) adding virus-inactivating chemicals to the IgG plasma fraction collected in step (e);

g) subjecting the plasma fraction resulting from step (f) to cation exchange wherein virus-inactivating chemicals added in step (f) are removed by adsorbion of IgG to the cation exchanger, followed by release of IgG and collection of the released fraction;

h) concentration of the IgG plasma fraction collected in step (g);

i) formulation of the IgG plasma fraction concentrated in step (h); and

j) sterile filtration of the formulated IgG plasma fraction obtained in step (i).

The present method is advantageously used for the production of IgG from human plasma, and may be used to prepare IgG for medical and/or diagnostic applications. The fresh plasma used as starting material in the present method may e.g be cryosupernatant plasma that has passed a chromatographic step for adsorption of vitamin K dependent factors (FIX, prothrombin, FVII, FX); cryosupernant plasma from which the prothrombin complex has not been removed; plasma that has passed a gel filtration medium for removal of FVIII and that has passed a chromatographic step for adsorption of vitamin K dependent factors (FIX, prothrombin, FVII, FX); or plasma that has passed a gel filtration medium for removal of FVIII and from which the prothrombin complex has not been removed.

Thus, as compared to the above-discussed US Pat. No. 6,835,379, the novel process according to the invention is reduced by one step, namely a cation exchange step. The process is based on the finding that by carrying out the virus inactivation earlier in the process, removal of one cation exchange step was possible. Thus, the present inventors have found that the contaminants removed in the prior art directly after the anion exchange, wherein the flow-through from the anion exchange used to be applied directly onto a cation exchanger, are equally well removed according to the invention in the cation exchange step carried out subsequent to the virus inactivation to remove

chemicals, as evidenced by the equivalent quality obtained according to the invention. Especially as the two prior art cation exchange steps were carried out at different pH values, the results of the present invention were quite unexpected.

In an advantageous embodiment, the plasma is subjected to gel filtration. In one embodiment, the removal of euglobins is by gel filtration and precipitation. The gel filtration may e.g. be carried out using Sephadex™ (GE Healthcare Bio-Sciences, Uppsala, Sweden).

The purification and/or removal steps above are preferably run as chromatography. Appropriate separation media used in these steps are hydrophilic in the sense that they are able to expose surfaces carrying hydrophilic groups, such as hydroxy, amido etc, to the liquid sample containing IgG. Appropriate separation media may be found amongst those that are based on synthetic polymers and/or biopolymers (for instance polysaccharides) carrying hydrophilic groups as referred above. Depending on where in the process the media is to be applied they may be uncharged or may carry positively charged (e.g. ammonium groups) and/or negatively charged groups (e.g. carboxy groups and sulphonic acid groups).

Thus, in one embodiment, the albumin is removed by anion exchange, preferably on a weak anion exchanger. In a specific embodiment, the weak anion exchanger is DEAE Sepharose™ FF (GE Healthcare, Uppsala, Sweden). In this context, it is understood that DEAE refers to cross-linked dextran substituted with diethylaminoethyl groups.

In another embodiment, step (d) uses a strong anion exchanger, and preferably an anion exchanger comprises quaternary amine groups (Q groups). In a specific embodiment, the strong anion exchanger is Q Sepharose™ FF (GE Healthcare, Uppsala, Sweden).

Virus inactivation may be carried out be the skilled person in this field according to well known principles, preferably by adding chemicals which inactivate virus. Such chemicals are well known in this field, such as the solvent/detergent (S/D) system.

However, once the virus has been duly inactivated, such chemicals need to be removed. thus, in one embodiment, the virus inactivating chemicals are removed by adsorption of the IgG to a weak cation exchanger. In this embodiment, the virus inactivating chemicals will remain in the flow-through, i.e. in the fraction that passes through the chromatography column.

In a specific embodiment, the following chromatographic media are used:
step c): DEAE Sepharose™ FF;
step d): Q Sepharose™ FF;
step g): CM Sepharose™ FF.
Sephadex™ and Sepharose™ (GE Healthcare Bio-Sciences, Uppsala, Sweden) are based on cross-linked dextran and agarose, respectively. CM refers to carboxy methyl groups.

The concentrating according to (c) is preferably performed immediately after albumin removal (for instance after step c as defined above) by ultrafiltration to less than or equal to the volume of the starting plasma.

In a preferred embodiment, the concentration of step (h) comprises ultrafiltration and diafiltration. In one embodiment, the method also comprises lowering, after step (g), preferably in step (h), of the ionic strength to 0.5 mS $\pm$ 0.1.

Detailed description of the drawing

Figure 1 shows schematically an illustrative embodiment of the method for producing IgG according to the invention. As appears from this figure, the sequence of steps has been reduced as compared to the prior art. As shown in example 1 below, the reduction of steps quite unexpectedly resulted in results which were well comparable to the comparative Example 2 in terms of yield and ACA.

EXPERIMENTAL PART

The present examples are given for illustrative purposes only, and should not be construed as limiting the invention as defined by the appended claims.

Example 1 – IgG production according to the invention

Materials / Investigated units

Columns:	BPG 100/500	GE Healthcare
	XK 50/30	GE Healthcare
	XK 50/20	GE Healthcare
Instruments:	BioProcess™Engineering	GE Healthcare
	BioProcess™Engineering	GE Healthcare
	Centrifuge, Cryofuge 5500I	Heraeus
	Pump Watson Marlow 505 S	Christian Berner
	Pump Watson Marlow 504 U	Christian Berner
	Recorder, Rec 102	GE Healthcare
	Monitor, UV-1	GE Healthcare
	Ultra filter, Pellicon	Millipore
	Cassette, Minisette 50	Filtron
	Water bath, grant SE 35	Grant
	pH meter, PHM 210	Radiometer
	Conductivity meter, CDM 210	Radiometer
	ÄKTA™FPLC	GE Healthcare
	Superdex™ 200 10/300 GL	GE Healthcare

	Phast™System	GE Healthcare
	PhastGel®Gradient 8-25	GE Healthcare
	Phast Gel®Native buffer Strips	GE Healthcare
	PhastGel™Blue R	GE Healthcare
	Spectrophotometer, Ultrospec 3000	GE Healthcare
	Stirrer, MR 3002	Heidolph
Resins:	Sephadex™G-25 M	GE Healthcare
	DEAE Sepharose™Fast Flow	GE Healthcare
	Q Sepharose™Fast Flow	GE Healthcare
	CM Sepharose Fast Flow	GE Healthcare
Chemicals:	Sodium acetate	Merck
	Sodium hydroxide	Merck
	Acetic acid	Merck
	Glycine	Merck
	Sodium chloride	Normapur
	Hydrochloric acid	Merck
	Tri-n-butyl phosphate	Merck
	Triton X-100	-
	Sucrose	BDH

Chemicals, analyses:	Tris(hydroxymethyl)aminomethan	Merck
	Substrate S.2288	
	Sodiumdihydrogenphosphate Monohydrat	Merck
	di-Sodium hydrogenphosphate	Prolabo
	Copper(II) sulphate pentahydrate	Merck
	Potassium sodium tartrate tetrahydrate	Merck
	Potassium iodide	Merck
	Methanol	Merck
	Glycerol	Merck
Starting material:	Human plasma (fresh frozen)	Uppsala Akademinska sjukhus ref. Helena Lööf

Analyses done on the final product

Protein concentration: Biuret method

Protein distribution : Superdex™ 200 10/300 GL
for detection of polymers, aggregates, dimmers and fragments.

Gel electrophoresis: PhastGel™ Gradient,
Native Buffer strips,
native-PAGE Coomassie staining

Proteolytic enzyme activity: Substrate S-2288, Chromogenix

1% IgG solutions were used in all in-house analyses except for the concentration determination.

The reference used in the proteolytic enzyme activity test was 1% Gammonativ, Octapharma.

Anti-complementary activity (ACA) was performed at BioProducts Laboratory, UK, according to the European Pharmacopoeia.

Criteria for acceptance; European Pharmacopoeia recommendations:

ACA, Monograph 2.16.17

The consumption of complement is not greater than 50% (1 CH_{50}/mg of IgG).

Protein distribution, Monograph 6.20.4

Monomers and dimers not less than 90% and aggregates and polymers not represent more than 3 % of the total area.

There are no recommendations from the authorities in terms of the level of proteolytic enzyme activity. The results are compared to the results obtained for the reference.

Results

Results obtained according to the invention, when running the virus inactivation step (f) using solvent-detergent (S/D) directly after step (d) on the anion exchanger Q Sepharose™ Fast Flow, instead of after CM Sepharose™ Fast Flow as in the prior art.

Table 1: Results in terms of purity

Exp. no	Purity Superdex 200 10/300 GL %	Purity Gel electrophoresis %

1.	~90	100
2.	98.5	100
3.	96.3	100
4.	99.7	100
6.	99.3	100
8.	99.0	100
9.	100	100
10.	99.7	100
11.	99.3	100
<x> ± SD	99.0 ± 1.2	

Table 2: Results from determination of proteolytic enzyme activity and ACA.

Exp. no	Proteolytic enzyme activity, U/L	Proteolytic enzyme activity, U/L, Reference	ACA CH_{50}/mg IgG	Comments
1.	3.3	4.8	-	
2.	3.2	4.8	0.38	
3.	4.3	4.4	0.40	
4.	3.0	4.4	0.49	
5.	1.7	4.4	0.46	
6.	1.8	4.4	0.38	-

As appears from the above, the level of the proteolytic enzyme activity obtained according to the invention is lower compared to the reference. The results from determination of ACA are all within the specifications. Normal level of ACA in products produced according to the original method is about 0.6-0.9 CH_{50}/mg IgG.

As appears from the above, when reducing the number of steps in the plasma process, the cost and time consumption will be reduced as compared to the prior art. Another advantage is that fewer steps increase the yield (data not shown).

Example 2 – Comparative example

625 L of thawed plasma is buffer exchanged into a 0.005 M NaAc (sodium acetate) pH 7 on a column of diameter Di=800 mm and bed height of H=600 mm, packed with Sephadex™ G-25 C. The flow is more than 100 cm/h, preferably 300 cm/h corresponding to a flow rate of 1500 L/h.

The eluted plasma is collected in a tank and 1M acetic acid is added during stirring until the pH 5.2 is reached. The plasma is left standing without stirring for 4-12 hours at a temperature of 4-10°C. After standing the formed euglobulin precipitation is removed by centrifugation.

The plasma is adjusted with 1M NaAc, pH 5.2 to a final ionic strength of I=1.4 mS (Range I=1.30 - 1.50). The pH shall be between 5.15 - 5.25.

The plasma is applied in 6 cycles, 25 - 30 g of albumin per litre gel, on a column of Di=1000 mm and H= 150 mm, packed with DEAE Sepharose FF and equilibrated with 0.020 M NaAc, pH 5.2. The linear flow rate is more than 60 cm/h, preferably 120 cm/h corresponding to a flow rate of 942 L/h. In the equilibration buffer the IgG will pass the column whilst the albumin is adsorbed. After 3 cycles the column is washed with 1.7 Vc (Vc =bed volume) of 0.15 M NaAc pH 4.0 + 0.5 M NaCl, 0.5 Vc of 0.5 M NaOH and 1.7 Vc of 0.15 M NaAc pH 4.0.

The IgG fraction of about 2350 L is concentrated by ultrafiltration to a final volume less or equal to 625 L, preferably 400 - 500 L. The procedure shall be started at the latest, when the whole fraction is collected from the DEAE Sepharose™ column.

The IgG solution is pH adjusted to pH 6.5 (6.45-6.55) with 1M NaOH and the ionic strength is adjusted to 1.40 mS (1.30-1.50 mS) by adding of WFI water (WFI = water for injection).

The IgG solution is applied in 6 cycles on a column of Di= 1000 mm and H= 150 mm, packed with Q Sepharose™ FF and equilibrated with 0.020 M NaAc, pH 6.5. Linear flow rate is more than 30 cm/h preferably 100 cm/h corresponding to 785 L/h. After 3 cycles the column is washed with 0.5 Vc of 0.5 M NaOH and 1.7 Vc of 0.15 M NaAc pH 4.0 The break through fraction containing IgG is directly adsorbed on the next column of Di= 800 mm and H=80 mm and packed with CM Sepharose™ FF. When the IgG fraction from all 6 cycles has been pumped through, the column is washed with 10 Vc of 0.01 M Glycine buffer, pH 7.0. The IgG is then eluted with 7 Vc of 0.1 M Glycine + 0.15 M NaCl pH 9.0.

The pH of the solution is adjusted to 4.0± 0.1 with 1M HAc (acetic acid) and concentrated by ultrafiltration to about 5% IgG.

Virus inactivation chemicals, Triton X-100 and TNBP, are added to the IgG solution during stirring. This mixture is transferred to the incubation tank for heat treatment at 30°C± 2°C for 4-16 hours. The ionic strength of the solution is adjusted to 1.40 mS by dilution with WFI and applied in 1 cycle on another column of Di= 800 mm and H= 80 mm, packed with
CM Sepharose™ FF and equilibrated with 0.020 M NaAc buffer pH 4.0. The linear flow rate is more than 40 cm/h preferable 80 cm/h corresponding to 400 L/h. After application the column is washed with 10 Vc of 0.01 M Glycine buffer pH 7.0 in order to remove the inactivation chemicals. The IgG is eluted with 7 Vc of 0.1 M Glycine + 0.15 M NaCl pH 9.0 at the same flow rate and adjusted to pH 4.0 with 1M HCl. The solution is then concentrated by ultrafiltration to 5% to 7% IgG and the ionic strength is adjusted by diafiltration to 0.5 mS±0.2 mS. Finally the solution is adjusted to 5.0%.

The solution is formulated to the following composition:

- Sucrose 1g/g IgG
- IgG 5%
- pH 4.0.
- ionic strength 0.5 mS ± 0.2 mS

After sterile filtration, filling and capping the solution is ready for delivery or storage.

ACA for different batches measured as defined above was found to be 0.5-0.7 CH_{50}/mg immunoglobulin.

CLAIMS

1. A method for producing IgG from plasma, which method comprises:
   a) buffering of fresh plasma;
   b) removal of euglobulins;
   c) removal of albumin followed by concentrating the IgG fraction so obtained;
   d) subjecting the plasma fraction obtained from step (c) to anion exchange and collecting the unbound plasma fraction comprising IgG,
   e) concentration of the IgG plasma fraction collected in step (d);
   f) adding virus-inactivating chemicals to the IgG plasma fraction collected in step (e);
   g) subjecting the plasma fraction resulting from step (f) to cation exchange wherein virus-inactivating chemicals added in step (f) are removed by adsorbion of IgG to the cation exchanger, followed by release of IgG and collection of the released fraction;
   h) concentration of the IgG plasma fraction collected in step (g);
   i) formulation of the IgG plasma fraction concentrated in step (h); and
   j) sterile filtration of the formulated IgG plasma fraction obtained in step (i).

2. A method according to claim 1, wherein the removal of euglobins is by precipitation.

3. A method according to claim 1 or 2, wherein the albumin is removed by anion exchange, preferably on a weak anion exchanger.

4. A method according to any one of the preceding claims, wherein the concentration according to step (c) is performed by ultrafiltration to less than the volume of the starting plasma.

5. A method according to any one of the preceding claims, wherein step (d) is followed by adjusting the pH to 4±0.1, and wherein the pH is maintained below 6.0 during the remaining steps of the method.

6. A method according to any one of the preceding claims, wherein step (d) uses a strong anion exchanger, and preferably an anion exchanger which comprises quaternary amine groups (Q groups).

7. A method according to any one of the preceding claims, wherein the virus inactivation of step (f) is carried out at a temperature of 30°C ± 2°C and for a duration of at least 4 hours.

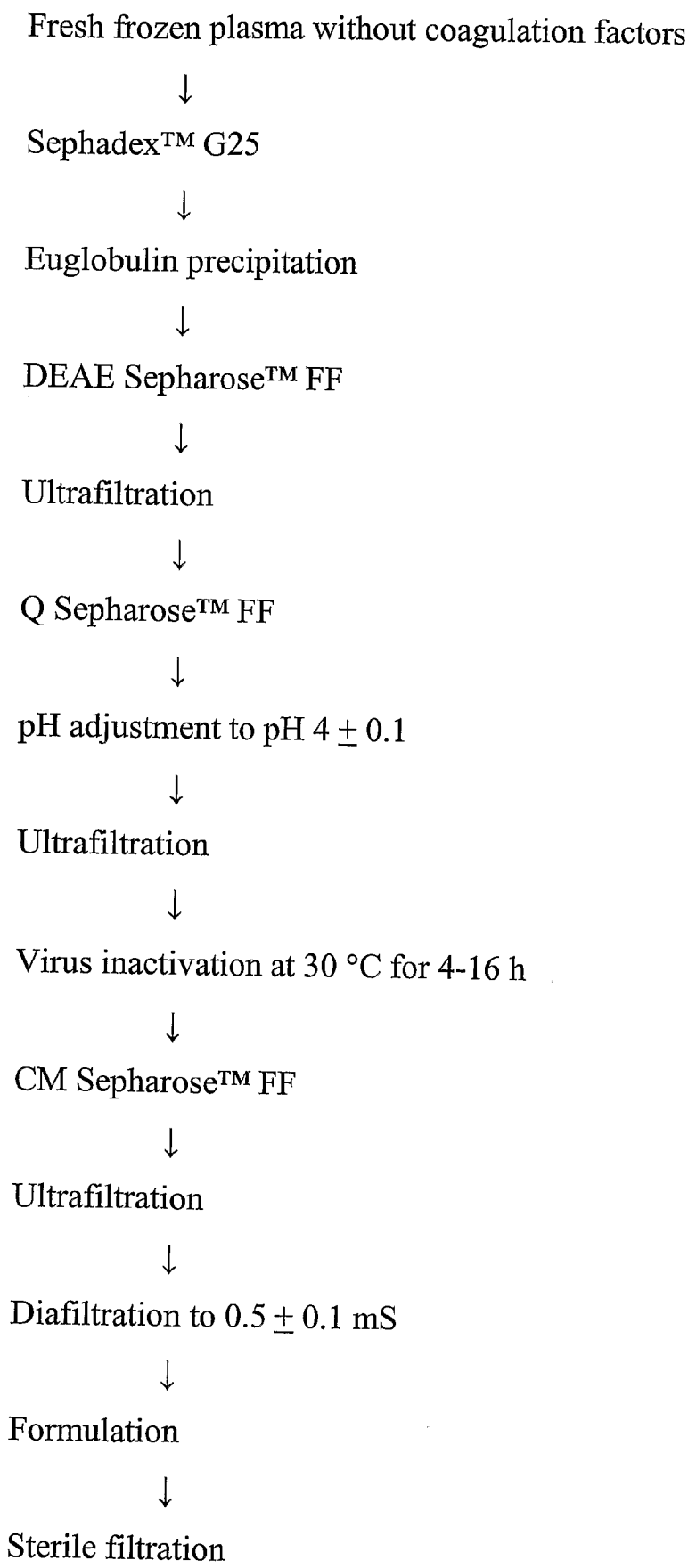
8. A method according to any one of the preceding claims, wherein step (g) is carried out using a weak cation exchanger.

9. A method according to any one of the preceding claims, wherein an acetate buffer is used in step (g).

10. A method according to any one of the preceding claims, wherein the concentration of step (h) comprises ultrafiltration and diafiltration.

11. A method according to any one of the preceding claims, also comprising lowering after step (g), preferably in step (h), of the ionic strength to 0.5 mS ± 0.1.

FIGURE 1


Fresh frozen plasma without coagulation factors
↓
Sephadex™ G25
↓
Euglobulin precipitation
↓
DEAE Sepharose™ FF
↓
Ultrafiltration
↓
Q Sepharose™ FF
↓
pH adjustment to pH 4 ± 0.1
↓
Ultrafiltration
↓
Virus inactivation at 30 °C for 4-16 h
↓
CM Sepharose™ FF
↓
Ultrafiltration
↓
Diafiltration to 0.5 ± 0.1 mS
↓
Formulation
↓
Sterile filtration

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2007/000486

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, MEDLINE, BIOSIS, EMBASE, TXTE, CHEM.ABS.DATA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 0172844 A2 (AMERSHAM PHARMACIA BIOTECH AB), 4 October 2001 (04.10.2001), claim 2	1-11
Y	WO 0076534 A1 (ALPHA THERAPEUTIC CORPORATION), 21 December 2000 (21.12.2000), page 13, line 24 - line 25; page 15, line 15 - page 16, line 8, claims 1,14,17	1-11
Y	US 6069236 A (MIRYANA BURNOUF-RADOSEVICH ET AL), 30 May 2000 (30.05.2000), column 3, line 61 - column 4, line 2, claim 1	1-11

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 August 2007	29 -08- 2007
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C07K 1/36 (2006.01)
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Cited literature, if any, will be enclosed in paper form.

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