

In a further aspect, the present invention relates to the implementation of a single step of ultrafiltration/diafiltration for the elimination or reduction of the levels of caprylic acid or salts of the same and/or the polyether or polymer of glycol used for viral inactivation in protein production processes.

Summary

Therefore, the present invention refers to a method for the preparation of a solution of immunoglobulins based on an initial solution of immunoglobulins with a purity greater than or equal to 96% in the presence of a polyether or polymer of glycol, characterised in that it comprises the steps of: a) adding caprylic acid or salts of the same to the initial solution at a concentration between 9 mM and 15 mM; b) adjusting the pH of the solution obtained in step a) to a pH between 5.0 and 5.2; c) incubating the solution obtained in step b) for the time and at a temperature necessary for the inactivation of enveloped viruses; and d) performing a step of ultrafiltration/diafiltration on the solution obtained in step c).

Described herein is a method for the preparation of a solution of immunoglobulins based on an initial solution of immunoglobulins with a purity greater than or equal to 96% in the presence of a polyether or polymer of glycol, characterised in that it comprises the steps of:

- a) adding caprylic acid or salts of the same to the initial solution;
- b) adjusting the pH of the solution obtained in step a);
- c) incubating the solution obtained in step b) for the

time and at the temperature necessary for the inactivation of enveloped viruses; and

d) performing a step of ultrafiltration/diafiltration on the solution obtained in step c).

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The method according to the present disclosure may also comprise a step of final formulation of the solution obtained in step d).

10 In the method according to the present disclosure, the initial solution of immunoglobulins is derived from fraction I+II+III, fraction II+III or fraction II, obtained according to the Cohn or Cohn-Oncley method, or from precipitate A or I+A or GG, obtained according to the
15 Kistler-Nitschmann method, or variations on the same,

which have been additionally purified to obtain an IgG purity greater than or equal to 96%. Preferably, the initial solution of immunoglobulins is derived from fraction II+III obtained according to the Cohn method or variations on the same, which has been subsequently purified by means of precipitation with PEG and anionic chromatography, as described in the document EP1225180B1. According to the present patent, any of the above fractions could be subjected to a precipitation procedure using PEG, followed by filtration in order to eliminate the precipitate and an additional purification step using an ionic exchange column (for example, a column with DEAE Sepharose). In all of these cases, the initial solution of immunoglobulins is derived from human plasma.

In the most preferred example, the initial solution of immunoglobulins is derived from fraction II + III obtained by procedures based on the Cohn method, which is additionally purified by any one of the methods described in the prior art to achieve an adequate level of purification to be subjected to the treatment with caprylate under the non-precipitating conditions of the present disclosure, i.e. a purity value greater than or equal to 96% (w/v) of IgG determined by electrophoresis in cellulose acetate, with an albumin content preferably less than or equal to 1% (w/v) with respect to the total proteins. Thus, the said initial solution of immunoglobulins is sufficiently purified, before and after the treatment with caprylate, for the route of therapeutic administration for which it is intended, so that no additional purification is required after the step with viral inactivation capacity of the present disclosure.

The immunoglobulins of the initial solution of the method according to the present disclosure can also be obtained by genetic recombination techniques, for example by expression in cell cultures; chemical synthesis
5 techniques; or transgenic protein production techniques.

In the most preferred example, the immunoglobulins mentioned in the method according to the present disclosure are IgGs. It is contemplated that the said IgGs
10 may be monoclonal or polyclonal. In the most preferred example, the IgGs are polyclonal.

It is contemplated that the polyethers or polymers of glycol of the present disclosure may be polyethers of
15 alkane or oxides of polyalkane, also known as polyglycols, and refer, for example, to derivatives of ethyl or ethylene and propyl or propylene, better known as polyethylene glycol (PEG) or polypropylene glycol (PPG), or equivalents of the same. In addition, the said reagents
20 must be compatible with the immunoglobulins in the sense that they do not compromise their stability or solubility and that, due to their size, they can be favourably eliminated by ultrafiltration techniques, or that, due to their lower toxicity, they are compatible with therapeutic
25 use of the immunoglobulins.

In a preferred example, the polyether or polymer of glycol is selected from polyethylene glycol (PEG), polypropylene glycol (PPG) or combinations of the same. Preferably, the
30 polyether or polymer of glycol is PEG, more preferably a PEG with a nominal molecular weight of between 3350 Da and 4000 Da, and most preferably a PEG with a nominal molecular weight of 4000 Da.

The content of the above-mentioned polyether or polymer of glycol in the initial solution of immunoglobulins is preferably between 2% and 6% (w/v), and more preferably
5 between 3% and 5% (w/v).

It is contemplated that it may possibly be necessary to adjust the concentration of the said polyether or polymer of glycol in the initial solution of immunoglobulins. The
10 said adjustment of the said polyether or polymer of glycol can be effected by diluting the initial purified solution of immunoglobulins and/or by adding the same.

According to the composition of the initial solution of
15 immunoglobulins, it is contemplated that, before step a) of the method according to the present disclosure, a series of steps of purification or adjustment of concentrations are carried out, such as, for example:

20 - adjustment of the concentration of immunoglobulins to between 1 and 10 mg/ml, more preferably between 3 and 7 mg/ml. This adjustment can be effected by any of the procedures known in the state of the art, for example by dilution or concentration of the protein to the
25 established range (determined, for example, according to total protein by optical density at 280 nm $E(1\%) = 13.8 - 14.0$ UA, by the Biuret method, by the Bradford method, or specifically by immunonephelometry), as the case may be. Therefore, in a preferred example, the initial solution of
30 immunoglobulins has a concentration of immunoglobulins preferably between 1 and 10 mg/ml, and more preferably between 3 and 7 mg/ml; and/or

- adjustment of the purity of the solution of immunoglobulins, which should preferably reach at least 96% of IgG with respect to the total proteins. This purification can be effected by techniques fully known to
5 a person skilled in the art, such as, for example, by precipitation with PEG, and filtration and subsequent anionic exchange chromatography (DEAE Sepharose).

In step a) of the method according to the present
10 disclosure, caprylic acid or salts of the same are added, preferably using a concentrated solution of the same, for example between 1.5M and 2.5M, to achieve a final concentration preferably between 9 mM and 15 mM.

15 In a preferred example, in step b), the solution obtained is adjusted to a pH between 5.0 and 5.2, more preferably to 5.1.

In a preferred example, in step c), the solution obtained
20 is incubated for at least 10 minutes, more preferably between 1 and 2 hours, and still more preferably 2 hours. In addition, the temperature at which the said incubation is carried out is between 2°C and 37°C, more preferably between 20°C and 30°C.

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In a preferred example, before step d) of the method according to the present disclosure, the content of polymers or aggregates with a high molecular weight in the solution obtained in the said step c) is less than or
30 equal to 0.2%, and more preferably less than 0.1%. This percentage of polymers or molecular aggregates of immunoglobulins with respect to the total proteins is determined by exclusion HPLC gel column according to the

optical density value at 280 nm. The said percentage of polymers or molecular aggregates of immunoglobulins can be evaluated, for example, using the analysis method described in the monograph on intravenous gammaglobulin of
5 the European Pharmacopoeia.

Preferably, the solution of immunoglobulins is clarified using depth filters before performing step d) of ultrafiltration/diafiltration.

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With respect to step d), it is contemplated, preferably, that the ultrafiltration/diafiltration in the method according to the present disclosure has initial steps of diafiltration and concentration by reduction of volume,
15 followed by the application of diafiltration at constant volume.

The ultrafiltration/diafiltration can be carried out on an industrial scale preferably by the method of simultaneous
20 dialysis and concentration, reducing the volume of product and diafiltering in turn, so that the consumption of reagents is somewhat lower and the process more efficient, taking account of the fact that the concentration of proteins is optimal and preferably less than or equal to
25 30 mg/ml. In any event, a person skilled in the art can easily determine the most appropriate and practical way of performing this step of ultrafiltration/diafiltration, choosing from among the various operating procedures known in the state of the art (for example,
30 dilution/concentration or diafiltration/concentration, diafiltration at constant volume, or modifications and combinations of the above).

The ultrafiltration/diafiltration membrane used in step d) of the method according to the present disclosure preferably consists of polysulphone, regenerated cellulose or equivalents, such as, for example, the membranes marketed under the brands Biomax® (Millipore, USA), Omega® (Pall, USA), Kvik-flow® (General Electric, USA). However, the molecular weight cut-off chosen for the membrane may vary depending on various factors, for example the manufacturer of choice. A person skilled in the art can easily determine the membrane of choice, which will be adjusted to the needs of each case depending, for example, on the concentration of caprylate and of the polyether or polymer of glycol in the solution to be processed.

Preferably, step d) of ultrafiltration/diafiltration is effected by means of a membrane with a molecular weight cut-off of less than or equal to 100kDa, more preferably of 100 kDa.

In the most preferable example, the ultrafiltration/diafiltration of step d) is performed in two phases:

a first phase in which the pH is adjusted to between 5.0 and 6.0 in order to reduce or eliminate most of the caprylate, and a second phase in which the pH is adjusted to less than 5.0, preferably to a pH of between 4.0 and 5.0, in order to reduce or eliminate most of the polyether or polymer of glycol.

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In a preferred example, in the first phase of the step of ultrafiltration/diafiltration, the diafiltration is performed using a diafiltration medium that comprises

alkaline salts of carboxylic acid, for example acetic acid, at a concentration greater than or equal to 5 mM approximately. In the most preferable example, the aforesaid diafiltration is performed using a solution of sodium acetate at a concentration greater than or equal to 5 mM adjusted to the pH mentioned above, i.e. between 5.0 and 6.0.

The number of diafiltration volumes to be performed in the first phase of step d) of ultrafiltration/diafiltration can be easily determined by a person skilled in the art according to the quantity of caprylate used initially and the acceptable final quantity. Preferably, at least three volumes of the diafiltration medium are used, the said diafiltration medium preferably being, as mentioned previously, a 5 mM solution of sodium acetate at pH 5.0-6.0. Preferably, in this first phase of the ultrafiltration/diafiltration, approximately 90% or more of the initial caprylate is eliminated, so that in this first phase the concentration of caprylate is reduced to approximately 1 mM or less.

In the second phase of step d) of ultrafiltration/diafiltration, the solution of immunoglobulins is diafiltered, preferably at constant volume.

Preferably, the diafiltration in the said second phase of the ultrafiltration/diafiltration is performed using a buffered solution that contains alkaline metal salts formed by acetate, phosphate or equivalents, or amino acids and/or polyols, for example glycine and/or sorbitol at the pH value indicated previously.

As in the case of the first phase of the diafiltration, in the second phase the number of dialysis volumes used to suitably reduce the polyether or polymer of glycol used in the method according to the present disclosure can be easily determined by a person skilled in the art taking account of the required reduction or elimination of the polyether or polymer of glycol. In a preferred example, the quantity of buffer to be exchanged in the diafiltration of the second phase of step d) of ultrafiltration/diafiltration is equal to or greater than six volumes. In the most preferred example, in the said second phase, the exchange is carried out according to the number of volumes of buffer necessary to obtain a reduction in the polyether or polymer of glycol equal to or greater than 100 times the initial content of the said polyether or polymer of glycol before beginning step d) of ultrafiltration/diafiltration.

Once the caprylate and the polyether or polymer of glycol have been reduced in step d) of ultrafiltration/diafiltration, in the final formulation step mentioned previously the solution can be adjusted to the desired final composition by adding the necessary excipients and/or stabilisers, so as to concentrate the product in order to achieve the final formulation. The addition of the excipients and/or stabilisers to be carried out after the final formulation can be effected directly by adding the said excipients and/or stabilisers in solid form or in a concentrated solution or, still more preferably, by means of diafiltration employing the necessary number of exchange volumes of a formulation solution to ensure the appropriate composition of the

final product.

In another example, the addition of the excipients and/or stabilisers is carried out by wholly or partially replacing the dialysis buffer solution of sodium acetate used in the second phase of step d) with a solution comprising the excipients and/or stabilisers, adjusted preferably to the same pH value of between 4.0 and 5.0 so that after the final concentration the immunoglobulin is already formulated.

A person skilled in the art knows which types of excipients and/or stabilisers must be added in order to achieve a desired stability. It is contemplated, for example, that the said excipients and/or stabilisers may be one or more amino acids, for example glycine, preferably at a concentration of between 0.2 and 0.3 M; one or more carbohydrates or polyols, for example sorbitol; or combinations of the same.

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Finally, the final concentration of immunoglobulins, preferably IgGs, is adjusted to a concentration suitable for its intravenous, intramuscular or subcutaneous use, which will be known to a person skilled in the art and may, for example, be between 5% and 22% (w/v). The said concentration is effected by any procedure known in the state of the art, for example concentration by ultrafiltration. It is contemplated that if the concentration of the immunoglobulins is effected by ultrafiltration, the said concentration may be carried out using the same membrane as in the previous diafiltration. Obviously, the three diafiltrations mentioned, as well as the concentration, may also be carried out using different

membranes.

The method according to the present disclosure also contemplates the possibility of introducing a step of
5 nanofiltration in order to increase the safety margin of the product. There are multiple phases in the procedure in which the product can be nanofiltered with commercially available filters (for example, Planova® and Bioex® made by Asahi-Kasei, DV® and SV4® made by Pall, Virosart® made
10 by Sartorius, Vpro® made by Millipore, or equivalents) with pore sizes from 20 nm or less and up to 50 nm, preferably with pore sizes of 20 nm or less, or even nanofilters of 15 nm can be used. The intermediate steps in which a nanofiltration step can be carried out are,
15 for example, in the initial solution of immunoglobulins; or in the material treated with caprylate after the step of ultrafiltration/diafiltration (once the caprylate and the polyether or polymer of glycol have been reduced); or in the material after concentrating and formulating the
20 solution of immunoglobulins, preferably IgGs (final product). A person skilled in the art will select the best option depending on, among other things, the pore size of the membrane, the filtration area required according to the time of the procedure, the volume of product to be
25 nanofiltered, and the protein recovery.

The final product obtained by the method according to the present disclosure complies in full with the criteria of the European Pharmacopoeia in relation to the content of
30 isohemagglutinins. However, the method according to the present disclosure also contemplates the option of including a step of selective and specific capture of anti-A and/or anti-B blood antibodies in order to maximise

their reduction. This step is preferably carried out using biospecific affinity resins, as has been described in the state of the art. For example, by using biospecific affinity resins with ligands formed by trisaccharides, a significant reduction in the level of isohemagglutinins can be achieved (Spalter et al., Blood, 1999, 93, 4418-4424). This additional capture may optionally be incorporated, at the discretion of the person skilled in the art, in any step of the method of the present disclosure, or may be done before or after carrying out the method of the present disclosure.

Therefore, with respect to the method for the preparation of a solution of immunoglobulins according to the present disclosure, in the most preferred example an initial solution of immunoglobulins with a purity greater than or equal to 96% of IgGs is used. This solution is adjusted to a concentration of IgGs preferably between 1 mg/ml and 10 mg/ml, and preferably between 3 mg/ml and 7 mg/ml, which contains (by addition in previous steps) or to which is added PEG to a concentration of $4 \pm 1\%$ (w/v). The pH of the solution is then adjusted to between 5.0 and 5.2 with acetic acid, and sodium caprylate is added (for example, using a concentrated solution of the said sodium caprylate). In the preferred example, the concentrated solution of caprylate is added to the purified solution of IgGs, slowly and with agitation. After adding all the caprylate calculated to bring the product to the final concentration of between 9 and 15 mM of caprylate, the final pH is then adjusted, if necessary, to between 5.0 and 5.2, and the solution is incubated preferably at a temperature of between 2-37°C, and more preferably at a temperature of $25 \pm 5^\circ\text{C}$, for at least 10 minutes, and

hermetically sealed. Another option is dosification in compatible rigid or flexible plastic containers, for example bags or bottles.

5 The dosified product goes through quarantine and visual inspection before being put into storage at a temperature between 2 and 30°C for conservation up to at least 2 years.

10 Moreover, as mentioned previously, the present disclosure also discloses for the first time the use of caprylic acid or salts of the same in the presence of at least one polyether or polymer of glycol for viral inactivation in protein production processes, in which the said polyether
15 or polymer of glycol and the caprylic acid or salts of the same are subsequently eliminated by means of ultrafiltration.

Preferably, the said proteins are selected from the group
20 of proteins that comprises immunoglobulins; albumin; coagulation factors such as factor VII, factor VIII and factor IX; and von Willebrand factor. Still more preferably, the said proteins are immunoglobulins. In the most preferred example, the said proteins are IgGs.

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The present invention will now be described in greater detail with reference to various examples of embodiment. However, these examples are not intended to limit the scope of the present invention, but only to illustrate its
30 description.

CLAIMS

1. Method for the preparation of a solution of immunoglobulins based on an initial solution of immunoglobulins with a purity greater than or equal to 96% in the presence of a polyether or polymer of glycol, characterised in that it comprises the steps of:

- a) adding caprylic acid or salts of the same to the initial solution at a concentration between 9 mM and 15 mM;
- b) adjusting the pH of the solution obtained in step a) to a pH between 5.0 and 5.2;
- c) incubating the solution obtained in step b) for the time and at a temperature necessary for the inactivation of enveloped viruses; and
- d) performing a step of ultrafiltration/diafiltration on the solution obtained in step c).

2. Method according to claim 1, characterised in that the said method also comprises a step of final formulation of the solution of immunoglobulins obtained in step d).

3. Method according to claim 1 or 2, characterised in that the initial solution of immunoglobulins is derived from fraction I+II+III, fraction II+III or fraction II, obtained according to the Cohn or Cohn-Oncley method, or from precipitate A or I+A or GG, obtained according to the Kistler-Nitschmann method, or variations on the same, which have been additionally purified to obtain a purity greater than or equal to 96% of IgG.

4. Method according to claim 3, characterised in that the

initial solution of immunoglobulins is derived from fraction II+III obtained according to the Cohn method or variations on the same, which has been subsequently purified by means of precipitation with PEG and anionic
5 chromatography.

5. Method according to any one of the preceding claims, characterised in that the initial solution of immunoglobulins has a concentration of immunoglobulins
10 between 1 and 10 mg/ml.

6. Method according to claim 5, characterised in that the initial solution of immunoglobulins has a concentration of immunoglobulins between 3 and 7 mg/ml.
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7. Method according to any one of the preceding claims, characterised in that the polyether or polymer of glycol is selected from polyethylene glycol (PEG), polypropylene glycol (PPG) or combinations of the same.
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8. Method according to claim 7, characterised in that the concentration of PEG in the initial solution is between 2% and 6% (w/v).

25 9. Method according to claim 7, characterised in that the concentration of PEG in the initial solution is between 3% and 5% (w/v).

10. Method according to claims 7 to 9, characterised in
30 that the PEG is PEG with a nominal molecular weight of 4000 Da.

11. Method according to any of the preceding claims, characterised in that in step b), the solution obtained is adjusted to a pH of 5.1.

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12. Method according to any one of the preceding claims, characterised in that in step c), the solution is incubated for at least 10 minutes at a temperature between 2°C and 37°C.

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13. Method according to claim 12, characterised in that in step c), the solution is incubated for 2 hours at a temperature between 20°C and 30°C.

15 14. Method according to any one of the preceding claims, characterised in that the initial solution of immunoglobulins has an albumin content less than or equal to 1% (w/v) with respect to the total proteins.

20 15. Method according to any one of the preceding claims, characterised in that the initial solution of immunoglobulins is derived from human plasma.

25 16. Method according to claims 1 to 15, characterised in that the immunoglobulins of the initial solution of immunoglobulins are obtained by genetic recombination techniques, chemical synthesis techniques or transgenic protein production techniques, or in cell cultures.

30 17. Method according to any one of the preceding claims, characterised in that step d) of ultrafiltration/diafiltration is carried out using a membrane of 100 kDa.

18. Method according to any one of the preceding claims,
characterised in that step d) of
ultrafiltration/diafiltration is carried out in two
5 phases:

- a first phase in which the pH is adjusted to between
5.0 and 6.0 in order to reduce or eliminate most of the
caprylate;
- 10 - and a second phase in which the pH is adjusted to a
value less than or equal to 5.0, in order to reduce or
eliminate most of the polyether or polymer of glycol.

19. Method according to claim 18, characterised in that
15 in the second phase of step d) of
ultrafiltration/diafiltration, the pH is adjusted to
between 4.0 and 5.0.

20. Method according to claim 2, characterised in that in
20 the step of final formulation, excipients and/or
stabilisers are added, selected from one or more amino
acids, one or more carbohydrates or polyols, or
combinations of the same.

25 21. Method according to any one of the preceding claims,
characterised in that the final concentration of
immunoglobulins is adjusted to a concentration suitable
for its intravenous, intramuscular or subcutaneous use.