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(54) Title: PURIFICATION METHOD

(57) Abstract: The present invention is related to a method for purification of pegylated GCSF. In particular, the invention employs a cation exchange chromatography with varying conductivity of wash buffers to separate low and high molecular weight impurities from the desired pegylated GCSF.

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PURIFICATION METHOD

RELATED APPLICATION

This application is related to and takes priority from Indian Provisional Application 1430/CHE/2013 filed 29th March 2013 and is herein incorporated in its entirety.

FIELD OF THE INVENTION

The present invention relates to a purification process for pegylated Granulocyte Colony Stimulating Factor (GCSF).

BACKGROUND OF THE INVENTION

Differentiation and proliferation of hematopoietic cells are regulated by glycoproteins referred to as colony stimulating factors (CSFs). Of the various CSFs, the Granulocyte Colony Stimulating Factor (GCSF) stimulates the proliferation of specific bone marrow precursor cells and their differentiation into granulocytes. When administered to mammals, GCSF promotes a dramatic increase in circulating granulocyte populations.

GCSF is one of several proteins produced by recombinant DNA technology for therapeutic use. Of the two types of GCSF clinically available, lenograstim, the glycosylated form of GCSF is expressed in mammalian cells, and filgrastim, the non-glycosylated form is expressed in *Escherichia coli* (*E. coli*). Proteins expressed by recombinant DNA methods in bacteria such as *E. coli*, are usually expressed as insoluble aggregates called inclusion bodies. These protein aggregates are separated, solubilized in the presence of protein denaturing agents, refolded and then purified by appropriate column chromatographic procedures.

To purify GCSF, many processes have been disclosed in patent and scientific literature that comprise ion exchange chromatography, chromatofocusing, reverse phase chromatography, hydrophobic interaction chromatography, and combinations of these and other methods.

WO05077973 teaches a method of using a reverse phase column for purification of human recombinant proteins and US2012093765 explains a two phase extraction system for the preparation of pure recombinant human GCSF.

US20080171857 describes a method for purifying GCSF by using a cation exchange and a hydrophobic interaction chromatography step.

WO2008096370 explains a two-step chromatography, cation exchange followed by size exclusion after a one step refolding of GCSF for its purification.

US20050159589 teaches a method for purification of GCSF by using Immobilised Metal Affinity Chromatography.

EP1200471 describes the use of hydrophobic interaction chromatography and hydroxyapatite column for the purification of GCSF.

The stability of GCSF can be improved and the immune response against these proteins reduced when these proteins are coupled to polymeric molecules (Rajan, et al., Protein Sci. 2006 May; 15(5): 1063–1075). Veronese et al, discusses the impact of PEGylation on biological therapies (BioDrugs. 2008; 22(5): 315-29). WO94/28024 discloses that physiologically active proteins modified with PEG (Poly Ethylene Glycol) exhibit reduced immunogenicity and antigenicity and circulate in the bloodstream considerably longer than unconjugated proteins.

Various methods of conjugating polypeptides like GCSF with polymeric moieties like PEG are well known and extensively described in the prior art. The preparation of glyco PEGylated GCSF, for example, is described in WO2005055946 and WO2006074467 is directed to the preparation of conjugates between GCSF and PEG moieties.

The prior art discussed above describe various ways which can be employed to purify GCSF. However the modification of polypeptides with polymeric moieties like PEG, causes significant shift in the chemical and physical properties of those polypeptides. Therefore, methods, which are useful for the purification of non-modified

polypeptides may not necessarily be effective in purification of their modified versions. For example, a pegylation process may generate low and high molecular weight impurities which may require optimization of purification process for their removal. Removal of such impurities poses a significant challenge in the development of methods for the purification of pegylated GCSF. The principle object of the present invention is to provide an improved method for purification of pegylated GCSF with effective separation of high and low molecular weight impurities.

SUMMARY OF THE INVENTION

The present invention discloses a method for purification of pegylated GCSF that effectively separates low and high molecular weight impurities from desired mono-pegylated GCSF by using a cation exchange chromatography.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG 1:is an illustration of a chromatogram from the procedure as described in Example 2. The line marked “Cond” represents the increase in conductivity in mS/cm. Peak A, represents the eluate (pegylated GCSF) obtained from cation exchange chromatography resin.

DETAILED DESCRIPTION OF THE INVENTION

GCSF is a human endogenous secretory protein which selectively induces the development of granulocyte committed progenitors from multipotent hematopoietic cells.

The term "Peg-GCSF" or “pegylated GCSF” refers to a GCSF protein which is covalently linked with one or more polyethylene glycol moieties.

The term "wash buffer" as used herein refers to a buffer that is used to wash or re-equilibrate the ion exchange resin, or to elute one or more impurities from the ion exchange resin, prior to elution of the protein of interest.

The term "low molecular weight impurities" as used herein refers to forms of protein with lower molecular weight than the desired protein which includes but is not limited to non-pegylated forms of the protein, fragmented forms of the protein.

The term "high molecular weight impurities" as used herein refers to forms of protein with higher molecular weight than the desired protein which includes but is not limited to more than one pegylated form of the protein.

In current invention provides a method of purification of pegylated GCSF by cation exchange chromatography wherein a combination of post load washes of varying conductivity are employed.

In an embodiment, the invention provides a method of purification of pegylated GCSF by separating low molecular and high molecular weight impurities from a composition comprising Peg GCSF using cation exchange chromatography comprising

a. Loading the said composition onto a cation exchange chromatography column
b. Washing the cation exchange chromatography column with 1st wash buffer solution with a buffer of low conductivity.
c. Washing the cation exchange chromatography column with 2nd wash buffer solution with a buffer of high conductivity.
d. Eluting the desired Peg GCSF molecule from the column

In an embodiment, the invention provides a method of purification of pegylated GCSF by separating low molecular and high molecular weight impurities from a composition comprising Peg GCSF using cation exchange chromatography comprising

a. Loading the said composition onto a cation exchange chromatography column
b. Washing the cation exchange chromatography column with 1st wash buffer solution with a buffer of low conductivity.
c. Washing the cation exchange chromatography column with 2nd wash buffer solution with a buffer of high conductivity.
d. Eluting the desired Peg GCSF molecule from the column

wherein the said wash buffer solution of low conductivity has a value that is less than about 2 mS/cm and wherein the said wash buffer of high conductivity has a value that is about 18 mS/cm to about 21 mS/cm.

In another embodiment the second wash step may be followed by additional wash steps.

Cation exchange chromatographic step mentioned in the embodiments may be carried out using any weak or strong cation exchange chromatographic resin or a membrane, which could function as a weak or a strong cation exchanger. Commercially available cation exchange support include a resin, but are not limited to, those having a sulfonate based group e.g., MonoS, MiniS, Source 15S and 30S, SP Sepharose Fast Flow, SP Sepharose High Performance from GE Healthcare, Toyopearl SP-650S and SP-650M from Tosoh, S-Ceramic Hyper D, from Pall Corporation or a carboxymethyl based group e.g., CM Sepharose Fast Flow from GE Healthcare, Macro-Prep CM from BioRad, CM-Ceramic Hyper D, from Pall Corporation, Toyopearl CM-650S, CM-650M and CM-650C from Tosoh. Alternatively, the support could be a monolithic column, disk or tubular, that performs the function of a cation exchanger. In embodiments of the invention, a strong cation exchange resin, such as SP-Sepharose® (GE Healthcare Life Sciences) is used. This resin is made using a highly cross-linked, 6 % agarose matrix attached to a sulfopropyl functional group.

The invention is more fully understood by reference to the following examples. These examples should not, however, be construed as limiting the scope of the invention.

EXAMPLE 1

Pegylation of GCSF

Purified GCSF and methoxy-Poly Ethylene Glycol Propionaldehyde are mixed in a molar ratio of about 1:5 at a pH pH of 5.0±0.2. The mixture is incubated for 7 to 9 hours and then quenched with 75 mM acetic acid.

EXAMPLE 2

Cation exchange Chromatography

The pegylated GCSF obtained from example 1 was loaded onto a cation exchange resin, pre-equilibrated with sodium acetate (1.6 mS/cm). This was followed by a first post load wash with 20 mM sodium acetate at a conductivity of 1.6 mS/cm followed by a second post load wash with 20 mM sodium acetate, 175 mM NaCl at a conductivity of 18-21 mS/cm. The bound protein was eluted using a 20 mM sodium acetate buffer containing NaCl at a conductivity between 30 -32.5 mS/cm.

Claims

We claim:

1- A method of purification of pegylated GCSF by separating low molecular and high molecular weight impurities from a composition comprising pegylated GCSF using cation exchange chromatography, comprising the steps of;

a. loading the said composition onto a cation exchange chromatography support

b. washing the cation exchange chromatography support with 1st wash buffer solution with a buffer of low conductivity.

c. washing the cation exchange chromatography support with 2nd wash buffer solution with a buffer of high conductivity and

d. eluting the desired pegylated GCSF molecule from the support

2- A method according to claim 1, wherein the said wash buffer solution of low conductivity has a value less than 2 mS/cm.

3- A method according to claim 1, wherein the said wash buffer of high conductivity has a value about 18 mS/cm to about 21 mS/cm.

4- A method according to claim 1, wherein the second wash step may be followed by additional wash steps.

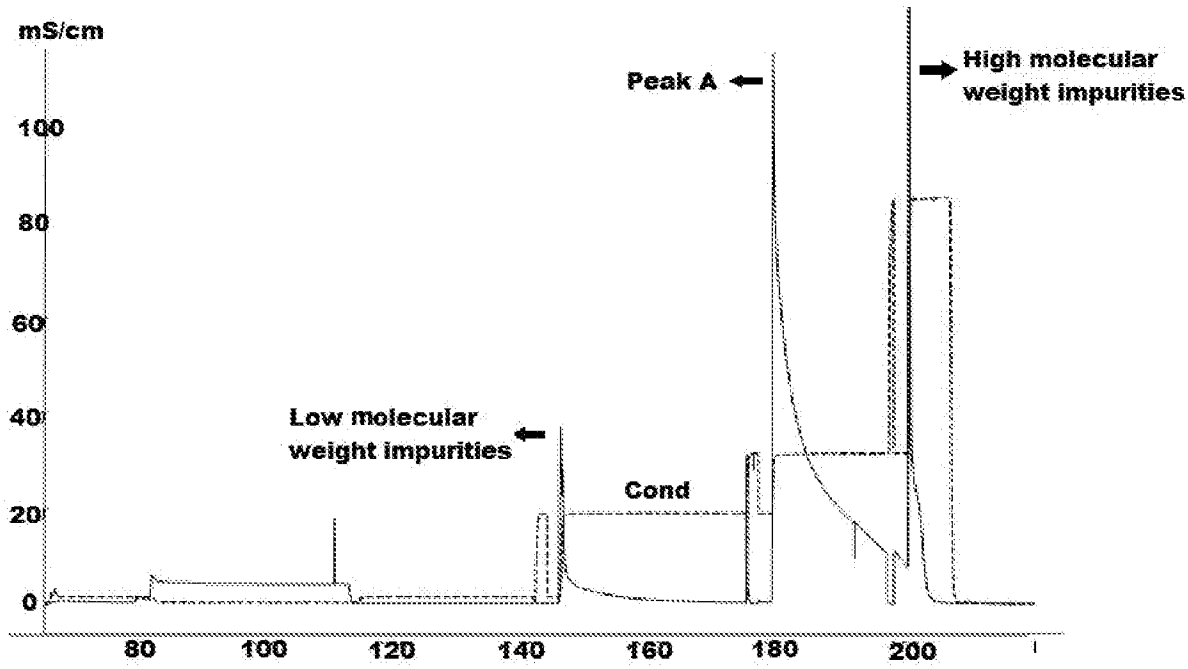
mS/cm
100
80
60
40
20
0
80
100
120
140
160
180
200
Peak A
High molecular weight impurities
Low molecular weight impurities
Cond

FIG.1