The present invention relates, in general, to a method of purifying recombinant human erythropoietin (EPO). The present invention also relates to a substantially pure EPO. The method comprises a differential precipitation, an hydrophobic interaction chromatography, various concentration and diafiltration steps, tandem anionic and cationic exchange chromatographies and molecular exclusion chromatography for the obtaining of pure EPO. The method does not comprise high performance liquid chromatography steps. The invention also comprises the EPO obtained according to the claimed procedure.
Abstract

The present invention relates, in general, to a method of purifying recombinant human erythropoietin (EPO). The present invention also relates to a substantially pure EPO. The method comprises a differential precipitation, an hydrophobic interaction chromatography, various concentration and diafiltration steps, tandem anionic and cationic exchange chromatographies and molecular exclusion chromatography for the obtaining of pure EPO. The method does not comprise high performance liquid chromatography steps. The invention also comprises the EPO obtained according to the claimed procedure.
DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS COMPREND PLUS D’UN TOME.

CECI EST LE TOME __1__ DE __2__

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JUMBO APPLICATIONS / PATENTS

THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE THAN ONE VOLUME.

THIS IS VOLUME __1__ OF __2__

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Methods of Purifying Recombinant Human Erythropoietin from Cell Culture Supernatants

Background of the Invention

Field of the Invention

Methods to obtain recombinant human erythropoietin (EPO) characterized by a sequence of tandem separation steps that includes differential precipitation, hydrophobic interaction, anionic exchange, cationic exchange and molecular exclusion liquid chromatographies. The EPO obtained by using the methods thus described.

Background Information

EPO is a glycoprotein that stimulates erythroblast differentiation in the bone marrow, thus increasing the circulating blood erythrocyte count. The mean life of erythrocytes in humans is 120 days and therefore, a human being losses 1/120 erythrocytes each day. This loss must be continuously restored to maintain a stable level of red blood cells.


The identification of the EPÓ production site in the organism was an issue of debate. Successive experiments led to identify the kidney as the main organ and peritubular interstitial cells as the synthesis site. See Jacobson, et al., Nature, 179, 633-4 (1957); Kuratowska, et al., Blood, 18, 527-34 (1961); Fisher, Acta Hematol., 26, 224-32 (1961); Fisher, et al., Nature, 205, 611-2 (1965); Frenkel,


Its therapeutical usefulness, however, has been limited due to the unavailability of a massive production method. The quantity and quality of the EPO obtained by the extractive systems known were insufficient. Recently, the use of recombinant DNA technology has made it possible to obtain large amounts of proteins. The application of these techniques to eukaryotic cells has allowed a large scale production of EPO. See patents US 5,688,679 (to Powell), US 5,547,933 (to Lin), US 5,756,349 (to Lin), US 4,703,008 (to Lin) and US 4,677,195 (to Hewick et al.).
Several techniques for the separation of glycoproteins such as EPO are currently available. Ultrafiltration, column electrofocusing, flat-bed electrofocusing, gel filtration, electrophoresis and isoelectrofocusing and some others chromatographic methods have been utilized for the purification of glycoproteins. The most widely used chromatographic techniques have been ionic exchange chromatography and adsorption chromatography.

The ionic exchange method is a separation technique by which the components of a solution are distinguished according to their different net charges and isolated by elution, either in stages or through the application of a continual gradient, with eluents of different ionic strength or pH. This method employs a gel or resin matrix, either of positive or negative charge, to induce binding or electrostatic adsorption of components with opposite charges. During desorption or elution, sample components are exchanged by ions present in the solution or buffer used to elute, or by a change in pH that alters the net charge of the molecule of interest.

Reverse phase adsorption chromatography involves separating the sample components according to their different polarities. Sample components are adsorbed through a resin composed of a silica matrix covered with an organic polymer by non-covalent bonding. The selective desorption of the components occurs afterwards by the elution with a non-polar solvent containing the eluent.

The separation techniques described above were utilized initially to separate relatively small hydrophobic or hydrophilic molecules. Their application to the purification of larger molecules, such as proteins, and specially complex proteins such as lipoproteins, nucleoproteins and glycoproteins, is more recent. Numerous publications illustrate the state of the art attained so far in protein separation.


Several specific methods for recombinant EPO separation have been recently reported. One of these methods consists in protein purification by anionic exchange chromatography with selective protease elimination, followed by reverse phase chromatography and filtration. See US patent 4,667,016 (to Lai et al.). This technique claims a yield of 16% EPO of unknown specific activity and purity.

Another method proposed for the separation of recombinant EPO consists in the application of reverse phase high pressure liquid chromatography (RP-HPLC) to a solution containing partially purified protein. See US patent 4,667,195 (to Hewick et al.). This method has been found irreproducible in practice. Moreover, the non-polar solvents commonly employed or recommended for protein and polypeptide separation by means of RP-HPLC, include reagents such as acetonitrile, difficult to remove from the protein of interest and potentially toxic for human beings. See Parsons, et al., Endocrinology, 114, 6, 2223-7 (1984). It should be noted, however, that ethanol and formic acid aqueous solutions for protein elution have also been used. See Takagaki, et al., Journal of Biological Chemistry, 5, 4, 1536-41 (1980).

Even though there is abundant information regarding the production of recombinant human EPO, a purification method yielding EPO adequate for its utilization in human beings has not yet been described. A suitable protein purification method should yield EPO over 99% pure and free of contaminants such as: aggregated material, b) degraded material, c) spurious proteins and d) proteases. A protein purity under 99% or the presence of any of the above mentioned contaminants might be toxic for human beings.

On the other hand, many of the methods proposed for EPO purification are not efficient when applied to industrial scale protein production. The RP-HPLC method employs expensive organic solvents, which increases
purification costs. In addition, organic solvents are more difficult to handle and contaminant to the environment. Other purification methods proposed are irreproducible in practice or have a low yield.

**Summary of the Invention**

The novel method of the present invention describes, in detail, a system for EPO purification whereby a high recovery of a product of high purity and quality is achieved. This product may be used without further purification to formulate pharmaceutical compounds as injectable products for use in human medicine.

An advantage of the claimed invention is the attainment of EPO protease free without any undesirable molecular variants such as aggregates, degraded material or molecules of unexpected isoelectric point values. The EPO obtained by the claimed invention is over 99% pure and could be utilized to prepare pharmaceutical formulations adequate for administration to human beings without any additional purification step. The EPO obtained by the claimed invention is a micro-heterogeneous protein comprising between five to eight isoforms with isoelectric points ranging between 3.0 and 4.5 and *in vivo* specific biological activity over 100,000 IU/mg protein measured by a $^{59}$Fe incorporation ex-hypoxic polycythemic mice assay and an EPO total mass spectrophotometric assay at 280 nm.

An additional advantage of the claimed invention is its low environmental impact. The method claimed is a clean process that does not employ separation steps based on RP-HPLC technology, thus avoiding the use of organic solvents that may be harmful to the environment.

Yet another advantage of the claimed invention is the non-exposure of EPO to stringent temperature conditions, harmful organic solvents or other solutions that may affect its biological activity or result in a toxic compound unsuitable for human use.

The following detailed description and examples illustrate the separation steps performed in the claimed method.
Brief Description of the Figures

Figure 1 illustrates polyacrylamide gel (SDS-PAGE) analysis of an EPO sample obtained following the method described after purification. In lanes 1, 4 and 7, molecular weight markers were loaded. In lanes 2, 3, 5 and 6, different amounts of pure EPO obtained according to the claimed procedure were run. The purity of the product obtained and the apparent molecular weight exceeding 34 kDa is coincident with the one reported for urinary human EPO as could be clearly observed.

Figure 2 illustrates a Western blot analysis of an EPO sample obtained according to the method described. Identity of the EPO produced is assessed, since it is recognized by a monoclonal antibody against human EPO. In lanes 1 and 2, a human EPO standard and molecular weight markers were loaded, respectively. EPO samples obtained according to the claimed method were loaded in lanes 3 to 5.

Figure 3 shows a SDS-PAGE analysis of a pure EPO sample obtained according to the method described, treated with glycanases. Molecular weight markers were loaded in lanes 1, 4 and 8. Lanes 2 and 7 correspond to untreated EPO. In lane 3, O-glycanase treated EPO was loaded; the presence of an O-glycosilation site is verified. In lane 5, N-glycanase partially digested EPO was loaded. The presence of 3 N-glycosilated molecules with molecular weights as expected for EPO can be verified. Lane 6 was loaded with EPO digested with N-glycanase, and the expected molecular weight for the wholly deglycosilated protein was obtained.

Figure 4 illustrates a survey of the isoelectric points in pure EPO samples produced according to the method described. EPO samples were run in lanes 2, 3 and 4, isoelectric point markers in lanes 1 and 5. The presence of isoforms corresponding to EPO are verified, showing an isoelectric point range of 3.0 to 4.5.

Figure 5 shows the purity of an EPO sample produced according to the method herein described using a reverse phase high performance liquid chromatography.
Figure 6 illustrate the purity of an EPO sample produced according to the method herein described using a molecular exclusion high performance liquid chromatography.

**Detailed Description of the Invention**

The present invention relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by a combination of the following steps: differential precipitation, hydrophobic interaction chromatography, diafiltration, anionic exchange chromatography, cationic exchange chromatography and molecular exclusion chromatography.

Preferred EPO producing recombinant cells comprise a vector which comprises a nucleotide sequence encoding the EPO polypeptide consisting of the amino acid sequence in SEQ ID NO:1, a viral promoter and a viral terminator. Preferred cells contain vectors which confer resistance to both methotrexate and neomycin-derived antibiotics. Preferably, the EPO nucleic acid molecule comprises the nucleic acid molecule described in Lin, "DNA Sequences Encoding Erythropoietin," U.S. Patent No. 4,703,008. Preferably, the viral promoter is an SV40 early promoter.

A preferred method of obtaining EPO from recombinant cells is culturing in media comprising insulin. Specifically, such culturing comprises separating the supernatant which comprises EPO and insulin from the host cells of the invention, concentrating the supernatant and freezing the concentrated product. Preferably, the culture media comprises between 0.5 mg and 20 mg insulin per liter of culture media.

The invention further relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by the following steps in order: (a) differential precipitation, (b) hydrophobic interaction chromatography, (c) diafiltration, (d) anionic exchange chromatography, (e) cationic exchange chromatography and (f) molecular exclusion chromatography.

The present invention relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by a combination of the
following steps: differential precipitation, hydrophobic interaction chromatography, 2 diafiltration steps, anionic exchange chromatography, cationic exchange chromatography and molecular exclusion chromatography.

The invention further relates to a method of purifying EPO which comprises treating cell culture supernatants comprising EPO by a following steps in order: (a) differential precipitation, (b) hydrophobic interaction chromatography, (c) diafiltration, (d) anionic exchange chromatography, (e) cationic exchange chromatography, (e') diafiltration and (f) molecular exclusion chromatography.

The differential precipitation step of the above and below-described methods and compositions comprises adding ammonium sulfate to said supernatant, followed by centrifugation.

The hydrophobic interaction chromatography step of the above and below-described methods and compositions comprises using an hydrophobic interaction matrix. Preferably said interaction matrix is Phenyl Sepharose 6 Fast Flow.

The anion exchange step of the above and below-described methods and compositions comprises using an anion exchange matrix. Preferably said anion exchange matrix comprises Q-Sepharose Fast Flow.

The cation exchange step of the above and below-described methods and compositions comprises using a cation exchange matrix. Preferably said cation exchange matrix comprises SP-Sepharose Fast Flow.

The molecular exclusion step of the above and below-described methods and compositions comprises using a molecular exclusion matrix. Preferably said molecular exclusion matrix is Sephacryl S-200 HP.

In another embodiment, the present invention provides a substantially pure EPO. Preferably, said EPO is produced by a combination of the following steps: differential precipitation, hydrophobic interaction chromatography, diafiltration, anionic exchange chromatography, cationic exchange chromatography and molecular exclusion chromatography. More preferably, said EPO has a purity of greater than 99% as determined by SDS-PAGE gel electrophoresis.

A preferred method of using the purified EPO of the present invention comprises lyophilization into a form suitable for injection into humans for
treatment of disease. Specifically, the preferred lyophilization procedure comprises placing the EPO into a pharmaceutical composition, loading the first EPO composition into a container, wherein said container is at a temperature equal to or less than -30°C; incubating said EPO composition at a temperature equal to or less than -30°C under atmospheric pressure for a time equal to or greater than 4 hours; incubating said composition at a pressure of equal to or less than 30 absolute microns for a time equal to or greater than one hour; and raising the temperature equal to or less than 3°C per hour until reaching at least 25°C, while keeping pressure values equal to or less than 5 absolute microns.

A preferred pharmaceutical composition for lyophilization comprises EPO, sugar, salts and human albumin. An especially preferred composition for lyophilization comprises EPO, mannitol, NaCl, NaH₂PO₄ and Na₂HPO₄•12H₂O.

The present invention is described in further detail in the following non-limiting examples.

**Examples**

**Example 1  Recovery**

7,920 g of ammonium sulfate were dissolved in 30 liters of sterile concentrated solution obtained from culturing CHO (Chinese Hamster Ovary) cells producing EPO. After addition of ammonium sulfate, the solution was stored at 4 °C for 24 hours. Many contaminant proteins precipitated while the EPO remained in solution. The product was centrifuged at 5,000 RPM for 10 minutes in a Sorvall centrifuge, using a HG4L rotor.

**Example 2  Hydrophobic Interaction Chromatography**

The material obtained from the previous step is chromatographed using an Hydrophobic Interaction matrix (Phenyl Sepharose 6 Fast Flow low sub.-Pharmacia) according to the following parameters:

1. **Equipment:**
A.  Pre-column:
1) Diameter: 14 cm
2) Bed height: 19 cm
3) Matrix:
   a) Q-Sepharose Big Bead (Pharmacia)
   b) Volume: 3,000 ml

B.  Column:
1) Diameter: 20 cm
2) Bed height: 19 cm
3) Matrix:
   a) Phenyl-Sepharose 6 Fast Flow low sub. (Pharmacia)
   b) Volume: 6,000 ml

2.  Solutions and buffers:
A.  Buffer A: 10mM NaH₂PO₄, pH 7.2
B.  Buffer F: 10mM NaH₂PO₄, 1.8 M (NH₄)₂SO₄, pH 7.2
C.  Buffer G: 150mM NaH₂PO₄, pH 7.2
D.  20% isopropyl alcohol
E.  0.5 N NaOH

3.  Material to be chromatographed:
A.  Ammonium sulfate supernatant resulting from previous example.
B.  Sample conditions:
   1) Volume: 30,000 ml
   2) Conductivity: 190-210 mS/cm
   3) pH: 7.2

To equilibrate and sanitize the pre-column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 volume of the column ("vc") (3 l) of H₂O; 1.0 vc (3 l) of NaOH 0.5N; 1.0 vc (3 l) of Buffer G and finally 1.5 vc (4.5 l) of Buffer F.
To equilibrate the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (6 l) of H₂O; 1.0 vc (6 l) of 20% isopropyl alcohol; 1.0 vc (6 l) of H₂O₂; 1.0 vc (6 l) of 0.5 N NaOH; 1.0 vc (6 l) of H₂O; 1.0 vc (6 l) of Buffer G and finally 1.5 cv (9 l) of Buffer F.

Once the pre-column and the column were equilibrated, the column was connected after the pre-column and the material to be chromatographed was loaded. Said loading was performed at 4°C, at a 19 cm/hour flow. Thereafter, the elution was performed at the same flow rate but at room temperature, and the solutions and buffers hereinafter detailed were passed through the columns in the following quantities and order: 2.5 vc (15 l) of Buffer F, (once this buffer has passed through, the pre-column was removed). Once the pre-column was removed, the chromatography was performed on the Phenyl Sepharose column on which a Buffer F-Buffer A gradient was applied starting from a 85:15 ratio of said buffers until 50:50 ratio of said buffers in a total volume of 10 vc (60 l) was reached.

When the gradient was finished, 1.5 vc (9 l) of Buffer F-Buffer A in a 30:70 ratio was passed through the column and finally 1.5 vc (9 l) of H₂O. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

**Example 3  Concentration and Diafiltration**

The fractions resulting from the previous example were concentrated and diafiltered according to the conditions described below:

1. **Equipment:**
   A. Peristaltic pump: Watson Marlow - Cat. N° 302S
   B. Tubing: Masterflex - Cat. N° 06402-18
   C. Concentrator: Prep Scale Millipore CDU F006LC

2. **Solutions and buffers:**
   A. 10mM Sodium Dodecyl Sulfate (SDS)
B. 1 mM Triton X-100
C. 0.1N NaOH
D. H₂O
E. Buffer A: 10 mM NaH₂PO₄, pH 7.2

3. **Material to be processed:**
   A. Selected fractions resulting from the previous example.
   B. **Sample conditions:**
      1. Volume: 15,000-30,000 ml
      2. Conductivity: 130-170 mS/cm
      3. pH: 7.2

The equipment was first cleaned, sanitized and equilibrated, and the following sequence of solutions and buffers were flowed through the equipment:

10 l of 10mM SDS; 40 l of H₂O; 10 l of 1 mM Triton X-100, 40 l of H₂O; 10 l of 0.1N NaOH; 40 l of H₂O and finally 5 l of Buffer A. The equipment was then ready to be used for concentration and diafiltration against Buffer A on the selected fractions, following the usual methodology.

The final volume of the concentrated product was between 2,000 to 3,000 ml, its conductivity was 1,100-1,550 μmS/cm and its pH was 7.2.

*Example 4 Anionic Exchange Chromatography*

The material resulting from the previous example was chromatographed using an anionic exchange matrix, as follows:

1. **Equipment:**
   A. **Column:**
      1) Diameter: 14 cm
      2) Bed height: 19 cm
      3) **Matrix**
         a) Q-Sepharose Fast Flow (Pharmacia)
         b) Volume: 3,000 ml
2. **Solutions and buffers:**
   A. Buffer A: 10 mM NaH$_2$PO$_4$, pH 7.2
   B. Buffer G: 150 mM NaH$_2$PO$_4$, pH 7.2
   C. Buffer N: 50 mM Acetic Acid, 500 mM NaCl, pH 4.0
   D. Buffer S: 50 mM Acetic Acid, pH 4.0
   E. 0.5 N NaOH

3. **Material to be chromatographed**
   A. Fractions selected from the hydrophobic interaction step, duly concentrated and diafiltered.
   B. Sample conditions:
      1) Volume: 2,000 to 3,000 ml
      2) Conductivity: 1,100-1,550 μS/cm
      3) pH: 7.2

   To equilibrate the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (3 l) of H$_2$O; 1.0 vc (3 l) of 0.5 N NaOH; 1.0 vc (3 l) of Buffer N; 2.0 vc (6 l) of Buffer S; 3.0 vc (9 l) of Buffer G; and finally 2.0 vc (6 l) of Buffer A.

   Once the column was equilibrated, the material to be chromatographed was loaded. Said loading was performed at room temperature at 39 cm/hour. Thereafter, the elution was performed at the same flow rate and temperature, and the solutions and buffers hereinafter detailed were passed in the following order: 1.0 vc (3 l) of Buffer A and 4.0 vc (12 l) of Buffer S. Thereafter, a Buffer S-Buffer N step (50:50) in a total volume of 1.5 vc (4.5 l) was performed.

   Once the step finished, 1.5 vc (4.5 l) of Buffer N was passed through the column. The selected EPO containing fractions, were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

**Example 5  Cationic Exchange Chromatography**

The material resulting from the previous example was chromatographed using a cationic exchange matrix, as follows:
1. **Equipment:**
   A. **Column:**
      1) Diameter: 14 cm
      2) Bed height: 19 cm
      3) Matrix
         a) SP-Sepharose Fast Flow (Pharmacia)
         b) Volume: 3,000 ml

2. **Solutions and buffers**
   A. Buffer D: 12.5 mM Na₂HPO₄, 4mM Citric acid, pH 6.0
   B. Buffer E: 12.5 mM Na₂HPO₄, 4mM Citric acid, 0.5M NaCl, pH 6.0
   C. 0.5 N NaOH

3. **Material to be chromatographed**
   A. Fraction selected from the previous example adjusted to pH 6.0 with NaOH cc and diluted until reaching a conductivity of 4,800 μS/cm (conductivity equal to Buffer D-Buffer E in a 93.5:6.5 ratio).
   B. Sample conditions:
      1) Volume: 5,000 ml
      2) Conductivity: 4,800 μS/cm (equal to Buffer D-Buffer E in a 93.5:6.5 ratio).
      3) pH: 6.0

To equilibrate the column, the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 vc (3 l) of H₂O; 1.0 vc (3 l) of 0.5 N NaOH; 1.0 vc (3 l) of Buffer E and finally 1.5 vc (4.5 l) of Buffer D-Buffer E in a 93.5:6.5 ratio.

Once the column was equilibrated, the material to be chromatographed was loaded. Said loading was performed at room temperature at 39 cm/hour.
Thereafter, the elution was performed at the same flow rate and temperature, and
the solutions and buffers hereinafter detailed were passed through it in the following order: 1.5 vc (4.5 l) of Buffer D-Buffer E in a 93.5:6.5 ratio. Thereafter, a gradient of Buffer D-Buffer E was applied starting from a 93.5:6.5 ratio of said buffers until a 50:50 ratio of said buffers in a total volume of 2.0 vc (6 l) was reached. Once the gradient was finished, 1.5 vc (4.5 l) of Buffer E was passed through the column. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

**Example 6  Concentration and Diafiltration**

The fractions resulting from the previous example were concentrated and diafiltered according to the following parameters and conditions:

1. **Equipment:**
   
   A. Peristaltic pump: Watson Marlow - Cat. No 302S
   B. Tubing: Masterflex - Cat. No 06402-18
   C. Concentrator: Prep Scale Millipore CDU F002LC

2. **Solutions and buffers:**
   
   A. 10mM Sodium Dodecyl Sulfate (SDS)
   B. 1 mM Triton X-100
   C. 0.1N NaOH
   D. H₂O
   E. Buffer B: 10 mM NaH₂PO₄, 0.15 M NaCl, 0.05 mg/ml Lactose, pH 7.2

3. **Material to be processed:**
   
   A. Selected fractions resulting from the previous step.
   B. Sample conditions:
      
      1. Volume: 6,000 ml
      2. Conductivity: 5,000-8,000 μSi/cm
      3. pH: 6.0
The equipment was first, cleaned, sanitized and equilibrated, letting pass through it the following sequence of solutions and buffers: 10 l of 10 mM SDS; 40 l of H₂O; 10 l of 1 mM Triton X-100, 40 l of H₂O; 10 l of 0.1N NaOH; 40 l of H₂O and finally 5 l of Buffer B. In this way, the equipment was ready to be used for the concentration and diafiltration procedures against Buffer B on the selected fractions, following the usual methodology.

The final volume of the concentrated product was 350-600 ml, its conductivity was 15,500-19,000 mSi/cm, the pH was 7.2, and the solution was stored at 4°C.

Example 7  Molecular Exclusion Chromatography

The material resulting from the previous example was chromatographed using a molecular exclusion matrix, as follows:

1. Equipment:
   A. Column:
      1) Diameter: 10 cm
      2) Bed height: 76 cm
      3) Matrix
         a) Sephacryl S-200 HP (Pharmacia)
         b) Volume: 6,000 ml

2. Solutions and buffers:
   A. Buffer B: 10 mM Na₂HPO₄, 0.15 M NaCl, 0.05 mg/ml Lactose, pH 7.2
   B. 0.5 N NaOH

3. Material to be chromatographed
   A. Fractions selected from the previous example, concentrated.
   B. Sample conditions:
      1. Volume: 350 to 600 ml
      2. Conductivity: 15,500-19,000 μSi/cm
3. pH: 7.2

To equilibrate the column the following solutions or buffers in the quantities hereinafter detailed were sequentially passed through it: 1.0 volume of the column ("vc") (6 l) of H₂O; 1.5 vc (9 l) of 0.5 N NaOH and finally 3.0 vc (18 l) of Buffer B. Once the column was equilibrated, 100 ml from the material to be chromatographed were loaded. Said loading was performed at room temperature at 27 cm/hour. Thereafter, the elution was performed at the same flow and temperature rates, and 0.75 vc (4.5 l) of Buffer B was passed through it. This procedure was repeated between four to six times, that is, until the material to be chromatographed was completely utilized. The selected EPO containing fractions were filtered under sterile conditions through a 0.22 μm pore membrane and stored at 4°C.

With this step the purification process was concluded. The EPO obtained has a purity degree superior to 99% and the entire purification process had a global yield of approximately 30%.

*Example 8 EPO Assays*

The EPO obtained in the previous example was assayed for identity and biological activity according to the following protocol.

In a denaturing SDS-PAGE gel the EPO was identified as a wide band of molecular weight as expected for EPO. See Figure 1. The band was recognized by monoclonal and polyclonal antibodies raised against human EPO in a Western blot assay as expected for EPO. See Figure 2. The treatment with glycanases proved the existence of the glycosidic chains in the extent and size as expected for EPO. See Figure 3. The EPO produced was shown to be composed of a series of species showing isoelectric points from 3.0 to 4.5 as expected for EPO. See Figure 4.

The complete amino acid sequence of the isolated protein, purified from the culture supernatant of transfected cell lines showed total homology with natural human erythropoietin whose 165 aminoacid sequence is as follows (SEQ ID NO:1):
The presence of the four glycosilation sites on the 165 amino acid chain, as well as the complex carbohydrate structure, and in particular, the sialic acid terminal residues, which characterize EPO were verified. These results were further supported by a biological activity assay of the produced protein by an ex-hypoxic polycythemic mice test, which showed complete concordance with the international EPO standard.

An EPO sample obtained according to the claimed method was submitted to a reverse phase and molecular exclusion high performance liquid chromatography analysis. In both cases, a purity over 99% was proved. See Figures 5 and 6.
The following table illustrates the recovery of each separation step corresponding to the claimed procedure.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>RECOVERY (%)</th>
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<tr>
<td>Hydrophobic Interaction Chromatography</td>
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<tr>
<td>Concentration and Diafiltration I</td>
<td>97</td>
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<tr>
<td>Anionic Exchange Chromatography</td>
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<tr>
<td>Cationic Exchange Chromatography</td>
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<tr>
<td>Concentration and Diafiltration II</td>
<td>95</td>
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<tr>
<td>Molecular Exclusion Chromatography</td>
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</tbody>
</table>

The following table illustrates the accumulated recovery of the purification sequence claimed in Claim 2.

<table>
<thead>
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<th>STAGE</th>
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<tbody>
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<td>Hydrophobic Interaction Chromatography</td>
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<td>Concentration and Diafiltration II</td>
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</tr>
<tr>
<td>Molecular Exclusion Chromatography</td>
<td>30</td>
</tr>
</tbody>
</table>

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All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.
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THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method for preparing a lyophilized recombinant human erythropoietin composition comprising:
   (i) providing a composition comprising:
       (a) recombinant human erythropoietin;
       (b) mannitol;
       (c) two or more salts selected from the group consisting of: NaCl, NaH₂PO₄, Na₂HPO₄, and mixtures thereof; and
       (d) human albumin;
   (ii) incubating the composition at a temperature equal to or less than -30 °C and at atmospheric pressure;
   (iii) exposing the composition to a pressure equal to or less than 30 microns; and
   (iv) raising the temperature of the composition at a rate equal to or less than 3 °C per hour until reaching a temperature of at least 25 °C while maintaining a pressure equal to or less than 5 microns.

2. The method of claim 1, wherein the composition comprises:
   (a) recombinant human erythropoietin;
   (b) mannitol;
   (c) NaCl, NaH₂PO₄, and Na₂HPO₄; and
   (d) human albumin.

3. The method of claim 1 or 2, wherein the composition of (ii) is incubated for a time equal to or greater than 4 hours.

4. The method of any one of claims 1 or 2, wherein the composition of (iii) is exposed for a time equal to or greater than 1 hour.
5. The method of any one of claims 1 to 4, wherein the composition of (ii) is incubated for a time equal to or greater than 4 hours, and wherein the composition of (iii) is exposed for a time equal to or greater than 1 hour.

6. A pharmaceutical composition made by the method of any one of claims 1 to 5.

7. A pharmaceutical composition for lyophilization comprising:
   (a) recombinant human erythropoietin;
   (b) mannitol;
   (c) two or more salts selected from the group consisting of: NaCl, NaH₂PO₄, Na₂HPO₄, and mixtures thereof; and
   (d) human albumin.

8. The pharmaceutical composition according to claim 7, wherein the composition comprises NaCl, NaH₂PO₄, and Na₂HPO₄.

9. A pharmaceutical composition for lyophilization comprising:
   (a) recombinant human erythropoietin;
   (b) mannitol;
   (c) NaCl, NaH₂PO₄, and Na₂HPO₄; and
   (d) human albumin.
Fig. 1. Polyacrylamide gel electrophoresis (SDS-PAGE)

Fig. 2. Western blot analysis
Fig. 3. SDS-PAGE analysis of EPO digestion with glycanases

Fig. 4. Determination of isoelectric point (isoelectric focusing)
Fig. 5. Reverse phase high performance liquid chromatography

Fig. 6. Molecular exclusion high performance liquid chromatography