Original Article

The Conductivity Change in the Course of Ion Exchange Chromatography: Effects on the Biological Activity and Isoforms of Human Recombinant Erythropoietin

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Abstract

Introduction: Recombinant human erythropoietin (*rhEPO*) has been produced in different conditions and cell lines. Identification, isolation, and purification of this protein from various sources have pivotal role in clinical applications. Hence, Clinical trials should be carried out for the identification of purity and aggregation of biological EPO. Moreover, the purification of EPO cooperates with various recommended processes has been manifested, but the strategies schemes (i.e., liquid chromatography (HPLC) and ion exchange chromatography (IEX) are often used in combination. In the current study, the quality of purification, biological modifications and the stability of rhEPO using various chromatography methods including HPLC and IEX have been assessed.

Materials and Methods: rhEPO was expressed in the Chinese hamster ovary (CHO) cells and purified by the general requirements for the quality control of biological products. For the assessment of the influence of IEX in the purity pattern of rhEPO, HPLC and biological analysis were performed for 3 samples.

Results: Our results revealed that the combination of 4 strategies represent confident methods for evaluating the quality of this biological medicinal product; moreover, purity and biochemical applications will yield to a relatively pure protein preparation. The activity of EPO was presented by monomeric isoform and high acid sialic purification in final product. Moreover, the determination of the biochemical reactions rate and their relationship tests were obtained by change in electrical conductivity with pH being 31.5 and 5, respectively.

Conclusion: Taken together, our results indicated that different purification process based on our results can increase the accuracy of rhEPO purification.

Keywords: Recombinant human Erythropoietin, Gel filtration, IEX, HPLC, Purification

1. Introduction

Erythropoietin, also known as EPO, hematopoietic, or hemopoietinis is an endogenous hormone(34 kDa) with half-life of 5 hours, existing in blood with the concentration of around 10 mU/ml [1]. Interstitial fibroblasts of the kidney and per sinusoidal cells of the liver are major responsible cells for producing the EPO. EPO stimulates the erythrocyte precursors in the bone marrow. This protein helps the proliferation and differentiation of immature blood cells in bone marrow as erythropoiesis and oxygen delivery to the

tissues of body[2]. Given that, the EPO administration to check health of the individual and sports ethics can consider the use of EPO and its analogue drugs as blood doping cases. Moreover, several studies revealed that the EPO could be used as therapeutic agent in different pathological conditions such ashaematological and oncological disorders, HIV infection, and perioperative therapies. The EPO receptor is expressed on non-erythroid cells such as endothelial and brain cells. The mutations of this protein is associated with emerging and developing different diseases. Erythropoietin could affect various biological processes such as hypertension, angiogenesis and cell survival [3].

Two forms of this protein, including epoitin alpha and beta are in market; nonetheless, both of endogenous and recombinant versions are available for utilization as therapeutic agents [4]. The improved life quality is the benefit of recombinant human EPO (rhEPO). It has been indicated that the detection of pure recombinant erythropoietin needs high cost processes [6, 11]. Erythropoietin is the most widely used recombinant drug across the world. Today, the rhEPO is used in the treatment of anemia, especially the treatment of anemia related to chronic kidney disease, dialysis and chemotherapy and anemia caused by HIV [4, 5]. Hence, developing new and effective purification platforms could contribute to more utilization of this form of protein in the clinical settings.

In this regard, a variety of experiments are used for purification of rhEPO for the purpose of increasing the purity solution by control requirement concerns such as separation based on size and ionization of molecule [6].

This well-designed and safe quality control results from stability testing, characterization and method validation by employing appropriate measures to ensure removing additional potential contaminants from products. Appropriate assessment does not have effect on biological potency. Changing some or all of these parameters such as a mixed mode ion-exchange chromatography matrix and/or utilization of the silica gel are ideal conditions and rapid purification strategies. Several columns using different details such as different types of sepharose and ultra-membrane filter as proper adsorption techniques can be performed for separation as a multiple-steps technique with high quality purification [7-9]. The combination of chromatographics, including various modes of filtration, high performance liquid chromatography (HPLC), ion exchange chromatography (IEX) technique and multiple complementary analytical technique are important for safe purification of various molecules [4, 8]. These analytical methodologies used for quality control (QC) of erythropoietin are used for drug development processes like Mab [10-12].

Dobrila et al., used three different high-performance liquid chromatographic (HPLC) techniques, i.e. affinity, ion exchange, and gel filtration chromatography without prior ammonium sulphate precipitation to purify polyclonal antibodies from bovine serum. The result showed that the rapid HPLC techniques were found to be very useful for the purification of polyclonal antibodies on a preparative scale means up to 25 mg of serum protein [13].

Sohraby et al., purified the recombinant factor VIII by production and purification of rabbit's polyclonal anti-body against factor VIII. Different techniques such as ion x change, affinity chromatographies, and ammonium sulfate precipitation of IgG or caprilic acid methods have been applied. The antibody was separated from the blood, purified and used to identify factor VIII [14]. Herein, we assessed the extended purification, release testing, and stability testing of rhEPO using various modes of chromatography including HPLC, IEX and gel filtration.

2. Materials and Methods

2.1 Cell culture

Chinese hamster ovary (CHO) were obtained from Pasture Institute, Iran. CHO cells expressed rhEPO were cultured in DMEM media containing 10% FBS (fetal bovin serum) for 48 h at 37°cwithout antibiotic. This media was substituted by DMEM media containing 5% FBS. The cell culture supernatant was harvested; then, the bottle adherent cell was separated by trypsination (for 2 times) and added to the solution. The insulin for removing FBS and PBS for solution washing was added. Cell culture filtration was done by 0/2 µm filter sterile.

This study was approved by the Pasteur Institute's ethics committee (Ethical code: IR.PII.REC.1395.011).

2.2 Gel Filtration

Gel filtration is a way to achieve the highest purity unaffected by buffer conditions, with limited volume capacity and sample size. Gel filtration was performed by the 60 ml of harvest that was loaded onto 185 ml of G-25Sepharosepre-equilibrated with sodium acetate (50 Mm) and tween 0/01 % buffer pH 5 at 3.7 ml/min flow rate until final fluid had pH: 5. Eluted result of gel filtration is well suited for use for other chromatographic techniques such as IEX. In this research, 60 ml elute including recombinant EPO and components as contaminant with 3.7 flow rate were described as sample. For this elution, sodium acetate at pH:5 and con:3-5 was used.

2.3 Ion Exchange Chromatography (IEX) and Ultrafiltrarion

XK50/2 chromatography column was prepared by 70 ml Q-Sepharose fast flow media pre-equilibrated with above-mentioned buffer at 20.1 mL/min flow rate. IEX is a technique which offers different solutions, either anion or cation exchangers. The pH of the performance can be modified to alter the charge characteristics of the sample components such as EPO. The column was equilibrated to the desired pH: 4 and 5. After passing the eluted fraction solution including EPO down the column, recombinant EPO was sequentially eluted with increasing concentrations of pH and concentration in the original buffer. Our experiment was based on modification of pH and electrical conductivity of different buffers. In this step, the first buffer (sodium acetate 50 mM, tween 20 0/01%) with pH: 5 and con: 3-5 was used as contamination and low sialic acid removing buffer, second buffer (sodium acetate 50 mM, tween 20 0/01%) with pH: 5, con: 3-5 (original buffer for eluting and EPO and sialicacid concentration increasing fraction) and third buffer was obtained with different electrical conductivity including 1: Sodium acetate 50 mM, tween 20 0/03% , con 30 with pH: 5, 2: Sodium acetate 50 mM, tween 20 0/01% , con 3-5 with pH: 4, 3: Sodium acetate 50 mM, tween 20 0/03% , and con 31.5 with pH: 5. Consequently, 75 ml of gel filtration elution was divided into 3 part solutions to be used for 3 columns separately. After any eluting, OD of every solution was measured. Fractions of these steps were analyzed by SDS-PAGE 12% for total protein and purity percentage. HPLC was used for eluting purposes. The ultrafiltration was concentrated by using a 10 KDa filter Amicon-Ultra (Millipore) into 15 ml elution.

2.4 HPLC Chromatography

The whole experiment was performed on HPLC instrumentation, consisting of 2.95 mL/min flow rate to an XK 26/70 column packed with 25.5 mL of high resolution gel filtration chromatography super grade × 200, prep grade media equilibrated with di sodium hydrogen phosphate buffer 70 mM, sodium hydrogen phosphate 2 H2O 30 mM, NaCl 50 mM buffer and tween 20 0/02% with con 15-17, pH: 7. HPLC samples were collected to test isoform and biological activity. The other techniques can be used

for the identification, assessment of purity and monomer, dimer and aggregation biological form of EPO using mouse injection. OD result manifested that the first pick belonged to aggregated form, second and third pick were dimer and monomer form of EPO respectively.

3. Results

Gel Filtration

This technique is based on separation by molecular mass, but here, it acts as a reducer of the conductivity. Hence, it provides a new possibility for separation of EPO from contaminant protein (not for purification). The decrease of pH from mobile phase to pH:4 confirmed that some contaminations were removed and also erythropoietin was deglycosylated. It means, the optimized pH for this application is 4. The result of gel filtration is depicted in Figure 1.

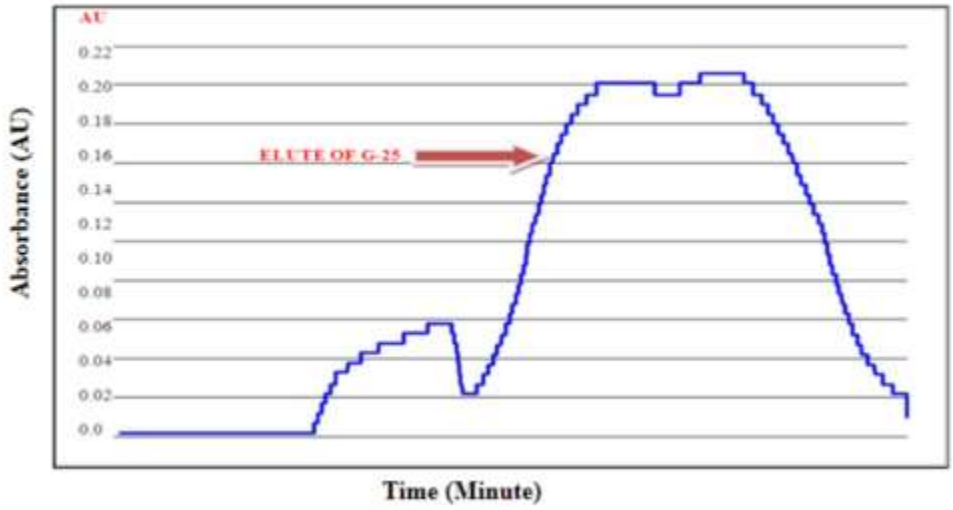

Figure 1. Gel filtration elution. The optimized pH is 4.

Ion Exchange Chromatography

In the anionic exchange chromatography of the basic form of erythropoietin, sialic acid was decreased and some other contaminants were increased. The mobile phase conductivity changing into 3 columns showed that the best purified fraction was eluted from anion exchange column, which was the sample with PH: 5 and 31.5 electrical conductivity and 27.7% purity (Table 1).

Table 1. Total protein and purity of final elution IEX

Sample	Total protein	Purity (SDS -PAGE)
Elution1	0.073	41.2%
Elution2	0.066	27.7%
Elution3	0.051	17%

HPLC Data Analysis

HPLC results which showed 3 biological EPO called aggregation, dimer and monomer (biological forms). These had 3 picks among which the sharpest one Elute of IEX column was used for HPLC (**Figure 2**). Finally, the output included belonged to monomer form with highest acid silic.

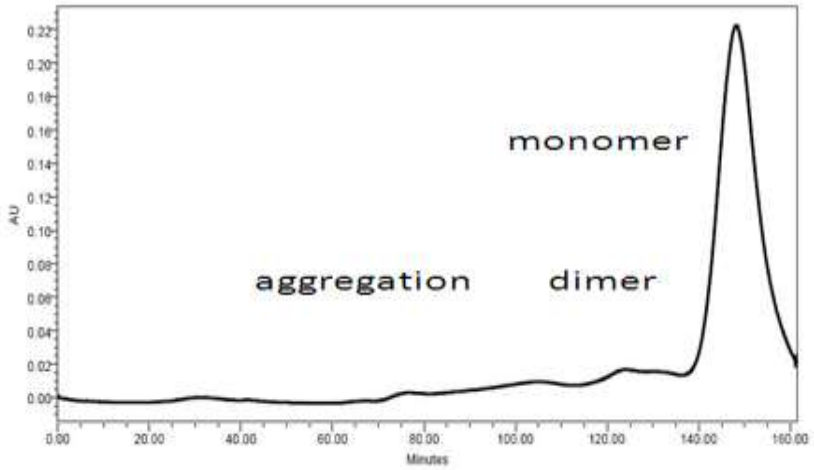

Figure 2. HPLC result with 3 picks including aggregation, dimer and monomer for EPO elution. The highest pick is for monomer form, with high acid sialic

4. Discussion

In the current study, we analyzed various procedures, including gel filtration, ultrafiltration, ion exchange chromatography and HPLC for erythropoietin purification. In this condition, the addition of minipore membrane was concentrated for HPLC chromatography. The combination of multiple purification steps yielded to a relatively pure EPO preparation.HPLC chromatography separated monomers, with the pick of electrical conductivity of 31.5, which was sharper than others. The obtained and increased biological forms with high acid sialic were in accordance with standard chart. Despite the wide use of chromatography for purification purposes, manufacturers of purification strategies are critical for researchers performing multiplex assays. This recombinant hormone in the roll of drug must undergo other methods such as ELISA, RIA and uHPLC for clinical applications in the world. Epoetin (known as human recombinant erythropoietin) was approved by the Food and Drug Administration (FDA) [15]. It has a wide range of actions such asstimulation of angiogenesis and promoting cell survival *via* activation of EPO receptors which resulted in anti-apoptotic effects on ischemic tissues. Given that, EPO caused a prompt decrease in plasma levels of renin and aldosterone [16]. Renal clearance studies suggested that EPO decreases renal proximal tubular reabsorption rate leading to activation of the tubuloglomerular feedback mechanism and a fall in glomerular filtration rate [17]. Thus, treatment with EPO may result in the suppression of endogenous EPO production through a decrease in intra-renal oxygen consumption [6, 11]. The cDNA clones of physiologic EPO have been isolated from different host cells, such as ovarian cells of Chinese hamster (CHO) and kidney cells from hamster cub [4, 5, 18]. A wide range of applications of EPO led to researchers attempt to discover or develop new *high-throughput* expression model, isolation and purification approaches which are associated with low costs and also could be easily introduced to clinical settings[6]For EPO, similar to other biologics, certain regulations quality control is necessary. The purification of target molecules from contaminants may require high identity, purity, strength, potency and safety strategies, like gel filtration and high performance liquid chromatography (HPLC). It is necessary to look at the possible detection of the contaminants and their deletion in the cell

extract and to add one or more steps to purification process [5, 7, 9].

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Nebija et al. assessed the characterization of the quality attributes including structural integrity, purity and stability of recombinant monoclonal antibody. The suitability of one-dimensional SDS-PAGE, under reducing and non-reducing conditions, and two-dimensional gel electrophoresis gel electrophoresis were valuated. They were analyzed for the recombinant monoclonal antibody, and trastuzumab. Results showed that it can bring about small pI difference between the normal and the recombinant form of the antibody[19].

Eivazi and colleagues used the affinity chromatography based on sepharose beads conjugated with protein A and ion exchange chromatography column for purification of mouse IgG2b. In the last protocol, direct ELISA was used to determine the titer of HRP conjugated rabbit IgG. This study documented that ion-exchange chromatography and affinity chromatography could be appropriate techniques for the purification of the antibody [11].

Bergmann-Leitner focused on two precipitation methods and two affinity chromatography-based separation methods and compared them for purifying rabbit and human. Also, human IgG was purified through different methods. In this regard, the significant lower titers of purified Igs, were observed. Meanwhile, no changes in the binding avidity (ED_{50} = 2.0 M for both sample) were observed[10].

Melkerson et al. purified the CMP-sialic acid lactosylceramide alpha 2,3-sialyltransferase (SAT-1), 40,000-fold to apparent homogeneity from rat liver Golgi through affinity chromatography twice on CMP-hexanolamine and once on lactosylceramide aldehyde-Sepharose 4B. Identification of the protein was detected through Western blot and immune protocol [9].

In a study, Gebauer et al. applied the lipid-enveloped viruses for the immunoglobulin

production by ion-exchange chromatography on Q-Sepharose Fast Flow (FF). The ionic strength of the starting buffer caused the elution of immunoglobulins, but did not affect immunoglobulin classes[8].

Miyake and colleagues employed a seven-step separation approach with a total EPO yield of 21%, which used size-exclusion, ion-exchange, and multimodal hydroxyapatite chromatography [20].

Utilization of affinity chromatography is another approach which could be used for purification of EPO. A very recent work, conducted by Kish et al. indicated that affinity chromatography which used cyclic peptide ligands, is a high efficacy purification approach for EPO [21].

5. Conclusion

In conclusion, this study is associated with some limitations. It seems that comparison between various platforms for purification of EPO are needed for obtaining more comprehensive results. Moreover, recent studies are carried out through new platforms such as MALDI-MS, and affinity chromatography using cyclic peptide ligands for evaluating the purification of EPO [6]. Thus, the comparison of the used platforms with new techniques could be related to devolving new and low costs purification approaches.

Conflict of Interest

The authors declare no conflict of interest.

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