Original Article

The effect of chromatographic buffering conditions on the human recombinant Erythropoietin production

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ABSTRACT

For producing recombinant erythropoietin, several cell lines are used. More common, CHO cell lines are employed to manufacture this protein. So, large amounts of impurities are produced during the process. Therefore, chromatography methods are used for purification. Optimizing the chromatography condition can improve the production process. Initially, the harvest was collected from cultured CHO cell lines. Then, the sample was applied to gel filtration, IEX, and high-resolution chromatography with different buffering conditions. The amount of obtained protein was measured by ELISA. SDS-PAGE, Capillary Zone Electrophoresis method, and peptide map were used to determine the effect of buffer condition on the eluted protein. The results indicated that the buffering condition of chromatography columns affected the quality and quantity of obtained proteins. Molecular weight and peptide maps of all samples were similar to the standard sample. CZE test showed that all samples were the same as the standard sample, except for one case. The results indicated the least change in chromatography conditions highly impresses our obtained proteins and led to significant variation in their quality and quantity.

Keywords: Chromatography, Human Recombinant Erythropoietin, peptide map, CZE test, optimization

Introduction

Given the increasing demand for recombinant therapeutic proteins, the production of these products has been one of the main drivers of the pharmaceutical industry's growth in recent years [1].

The recombinant erythropoietin, as a widely used therapeutic protein, has a significant portion of the pharmaceutical market. Therefore, achieving the optimum conditions, for the production and purification of this protein, is one of the critical challenges of the pharmaceutical industry [2].

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This protein has glycosylated portions that play a functional role in biological activity, so this recombinant protein produced routinely in Chinese hamster ovary (CHO). Consequently, the massive amounts of impurities are produced during the manufacturing process, because of the complexity of the cell culture conditions. Since human recombinant proteins require high purity, the purification and isolation of biologically active form of proteins from harvest mixture remain the most challenging tasks in biotechnology [3-5].

In the industrial phase of production of recombinant proteins, chromatography methods are the main methods for separation and purification purposes in the initial, intermediate, and final stages of purification processes. These processes have always considered as costly bottlenecks in the production of recombinant proteins. On the other hand, because of their different ionic strength and pH of buffers used in chromatographic methods, the biological activity of target protein can be changed by deformation, which results in the loss of valuable properties of the target protein. The complexity of

the issues raised in chromatographic methods is a significant factor in the rising cost of this class of therapeutic proteins [6, 7]. Investigating the practical factors in chromatographic techniques can be a suitable way to optimize and increase the efficiency of these methods. For this reason, in recent years, research on the improvement of chromatographic methods has been taken into consideration by researchers and companies producing these proteins. In conventional methods, gel filtration chromatography, ion-exchange chromatography, and high-resolution chromatography processes are applied for the purification of recombinant EPO [8, 9].

Objectives

The aim of this study was to investigate the effect of chromatographic buffering conditions on the quality and structure of the purified protein. The variables of buffering conditions are pH and conductivity. For this purpose, chromatography buffers with different pH, and conductivity were used to compare the results obtained from each process series to check the effect of the above-mentioned factors on the efficiency of the purification process. In this review, the flow rate, column dimensions, and temperature were constant.

Material and Method

Based on this study, a similar system of industrial chromatography techniques including gel filtration, ion-exchange chromatography, and high-resolution separation gel filtration (XK-16 column series - GE Healthcare, Uppsala, Sweden), which commonly use in the process of purification of recombinant erythropoietin, were exploited on a laboratory scale.

Sample collection

Recombinant erythropoietin (as a model) was obtained by culturing CHO cells (dihydrofolate reductase or DHFR). Briefly, the cell culture was conducted using Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Grand Island, USA) supplemented with human recombinant insulin (Gibco, USA) and after growth, expansion, harvesting phases, the collected supernatant from harvest was filtered by 0.2 μm filtration cartridge (Sartorius, Gottingen, Germany) and preserved at 4 °C.

Design of experiment

Initially, the supernatant was obtained from the homogenized cell culture. Then, it was divided into 15 equal volumes and each volume was subjected to the chromatography process with different buffering conditions. Each volume was investigated three times with each buffer so that triplicate replications were performed for both criteria. Then, the average of results was recorded for each sample. Initially, the gel filtration chromatography with Sephadex G-25 platform was used. At first, the column was balanced using sodium acetate buffer (PH: 5), and then harvest was applied to the column. Then, the column was washed with the same buffer, and proteins were eluted in containers. At this point, the buffer with different pH and conductivity was used, and the amount of protein obtained was measured.

In the next step, the sample obtained from the previous stage was homogenized and applied to Q-Sepharose ion-exchange chromatography column. After balancing the column, the protein was applied to the column. Afterward, the column was washed with 50 mM sodium acetate containing tween 20 (PH: 4). At this stage, proteins that are not absorbed by the resin were removed from the column. Subsequently, by changing the pH of the sodium acetate buffer (PH: 5), proteins that have a weak binding to the column were removed. At the end of the step, the column was washed with 0.3 M sodium chloride solution until the desired protein (rhEPO) eluted. By changing the pH and conductivity of the washing buffer, the yield of this step investigated, the purity and total protein content of the eluted were studied.

At the end of the process, a Superdex bed (G200) high-resolution chromatography column was used. After balancing the column, the sample was applied to the column. Di-sodium hydrogen phosphate buffer with variable pH and conductivity was used to measure the output of the column.

Structural study

The quality measurement of recombinant erythropoietin in the obtained sample was done by an ELISA commercial kit (EPO Elisa ROCHE). ODs of samples were measured using the ELISA Reader Stat Fox instrument (BioTek Instruments, Inc., USA). SDS-PAGE was used to check the molecular weight of the obtained proteins in each series. For this purpose, standard erythropoietin alpha (35 kDa) along with a molecular marker (14-116 kDa) was used. The samples were reduced by A12XR reducer buffer and placed on an SDS-PAGE gel with the standard molecular marker and standard erythropoietin. Then, after fixation of the gel with acetic acid, coomassie blue was used to visualize the bands. In the next stage, protein concentration was measured by spectrophotometry.

The erythropoietin peptide map was studied by the RP-HPLC method. For this test, samples were diluted with Tris 0.1 M to reach a concentration of 1 mg/ml. Then, 25 ml of the diluted sample was centrifuged at 5000 rpm for 5 minutes. After that, 0.2 ml of DI water was added and centrifuged again. After desalting, 5 μg of trypsin was added to samples and placed at 37 °C for 18 h. In the end, the samples were incubated at -20 °C to stop the enzyme reaction. After balancing the HPLC column, 50 μl of the sample and the standard were applied to the column. Samples and standard chromatograms were compared.

Finally, the glycosylation pattern of proteins was studied by the CZE method. To perform this test, the first CZE buffer preparation was performed as described in the European Pharmacopoeia method. The following components were added to a 100 mL volumetric flask: 0.582 g of NaCl (S1249 - Spectrum), 1.793 g of Tricine (part no. T5816 – Sigma), 0.820 g of sodium acetate (part no. 3470-01 - J.T. Baker), and ddi water was added up to 100 mL. This solution was filtered

through a 0.2 µm membrane using a Nalgene filter unit (MF75) and stored at 4 °C. Then, 500 µl of samples desalted two times by 250 µl WFI. Then, 100 µl of sample volume was injected into capillary electrophoresis. The sample diagram was compared with the standard model. The data were analyzed using the Pearson correlation test and regression analysis using SPSS version 16.0 (IBM). Statistical results with p-value <0.05 were considered significant.

Result

This study was carried out based on the protocols of recombinant erythropoietin purification of WHO Technical Report Series, No 937, 2006, Annex 4 [10]. According to the standard protocol, the acceptable range of pH and conductivity of buffer in the G25 chromatography column is 4.8-5.2 and 3-4.5, respectively. To investigate the effect of the factors on the efficiency of columns, buffers with different pH and conductivity were used in the mentioned range. The results showed that the amount of protein obtained in the gel filtration method of G25 under different conditions of buffer was 0.03 to 0.09 mg/ml. The results are as follows (Table 1).

In the following, according to the acceptable pH and conductivity buffering range mentioned in the manufacturing protocol for the ion exchange column, buffers with different conditions were used. The acceptable range is 4.8-5.2 and 30-35 ms/cm, respectively. The effect of different buffering conditions on the protein content during ion-exchange chromatography indicated that the output of the system varies between 0.16 and 0.33 mg/ml under different conditions. The results are as follows (Table 2).

Samples obtained from the previous step were applied to the gel filtration column with high-resolution (G200). The acceptable pH and conductivity range of buffer are 6.8-7.2 and 15-18 ms/cm according to the protocols for chromatography G200. So, by using buffers with different pH and conductivity, the efficiency of output was between 0.21 to 0.51 mg/ml. The results are as follows (Table 3). Eluted proteins obtained at each series of chromatography were electrophoresed by SDS-PAGE method to confirm the molecular weight of the protein (Figure 1). The results of the peptide map by RP-HPLC method showed that the chromatogram pattern of all samples was as same as the standard (Figure 2).

To study the glycosylation structure proteins, the CZE method was used. The concentration of the samples was 1.6 to 2.1 mg/ml. The isoform pattern of the samples was compared with the standard recombinant erythropoietin isoform (Figure 3). The results obtained from the comparison of the sample pattern with the standard model showed that sample number 3 has a peak beyond the acceptable range. The other samples had an acceptable profile. The results are represented in Table 4.

Statistical analysis

Statistical analysis of the results showed that there is a significant relationship between buffer conditions (pH and conductivity) and process output (P<0.001).

Discussion

The biological active groups of proteins are usually at the surface of the protein and, due to the sensitivity of these portions to the physical and chemical agents, the buffering conditions of the chromatographic methods can affect the structure of these proteins. So, the development of purification processes for therapeutic proteins has become a prime challenge in the industrial production of recombinant proteins. Achieving maximum purity over a short time has been considered as an important goal in the purification processes [11].

To optimize different chromatographic methods, proper process recognition, and analysis of the variables involved in each process are considered as important criteria. In studies over the past several years, many investigations have been done on increasing the efficiency of recombinant erythropoietin production. Most of the researches done in the field of production have focused on the upstream sections, while the study of downstream processes, especially the chromatographic methods were less relevant or limited to a single chromatographic method [12, 13]. Also in these investigations, the studies focused on one factor at a time (Changing One factor at a time), and therefore, the studies have often failed to generate effective results [14].

In the study by Choi in 2007, increasing the production of erythropoietin in CHO cells was evaluated, and the data showed that the optimal and stable pH for the cell line could increase the production. To this end, 40 mM of sodium lactate was added to the culture medium. The result showed that, although the growth rate decreased by about 22% compared to the control culture, due to a reduction of 52% in lactate production and pH stabilization during the production process, the final concentration of accumulated rEPO was increased 2.7-fold. Therefore, adding 40 mM sodium lactate had positive effects on EPO production.[15]

In another study by Arakawa et al. (2009), they examined the method of size exclusion chromatography. This method, despite its extensive application in the field of purification, has certain limitations. One of these limitations is the absorption of the target protein by resin. Therefore, in this study, the effect of NaCl as the mobile phase of gel filtration chromatography on the elution time of chymotrypsinogen and ovalbumin was investigated. So, the gradient concentration of 0 to 0.4 M NaCl was used. The results indicated that, with the rise of the buffer concentration in the pH=7, chymotrypsinogen gained a positive charge. So, its elution time decreased. On the other hand, ovalbumin got a negative charge. Thus its elution time increased [16].

In a report presented by Cabo-Calret et al. (2014), the effect of changing concentrations and flow rates of buffer on the chromatographic process were investigated. In this study, the modified percentage of the mobile phase was studied in different flow rates for elution of sulfonamides. The results showed that by increasing the percentage of modifiers (acetonitrile) from 5% with flow rate of 5 mL/min to 30% with flow rate of 1 mL/min, the analysis time reduced from 100 minutes to 22 minutes [17].

In a study published by Huz (2014), variable factors of chromatography such as column length and column pressure were investigated at different temperatures, and the results showed that achieving an optimum point in these three variables resulted in improved performance of gas chromatography [18].

Another research has been done to generate chromatographic simulation software, which has led to the design of the software. For example, DIONEX Company provided Virtual Column software to identify, examine, and analyze factors involved in the ion-exchange chromatography process. So, it can improve the results of chromatography methods by simulation of process on a laboratory scale [19].

Another software is provided by Ypso-Facto Company for modeling HPLC and LPLC processes for pharmaceutical use. Chrom-Work software can simulate the processes with high speed and accuracy [20]. Another software is ACD/AutoChrom, which was provided by Advance Chemistry Development, Inc. This software simulates an optimal method by checking the temperature and gradient buffering [21].

To achieve high purity, several chromatographic methods are used in the industrial phase of the production of recombinant erythropoietin. Also, As each chromatographic method has a buffer with different pH and conductivity, recombinant proteins undergo severe conditions, which results in a significant loss in the amount of the protein. In this study, by examining the factors effective in chromatography method in purification processing, the amount of purified protein, and the optimal conditions for improving the methods were studied [22-24].

The results of the obtained protein from the G25 column indicated that by using buffers No. 9 and 5, the protein content received during the three experiments was 0.09 and 0.08 mg/ml, respectively. However, the output rate of the process was 0.02 mg/ml by using buffer No. 3. The results showed that in the ion-exchange column, the highest concentration of protein (0.33 mg/ml) was obtained by using buffer 1 and the lowest amount was gained by using buffer 15 (0.16 mg/ml). Also, in column G200, the highest protein content was obtained by washing with buffer 2, while buffer number 7 had the lowest output performance.

The results represented that, the minor changes in pH and conductivity of buffers, led to a significant change in the amount of protein obtained from each chromatography step. Therefore, to accelerate and improve the purification process, it is essential to simulate chromatography methods to provide the best conditions for achieving favorable results [25, 26]

So, by using other chromatographic methods such as affinity chromatography instead of ion-exchange chromatography and applying the simulator software, the chromatographic methods can be studied and optimized. For this reason, using alternative methods and comparing them with current methods are suggested to achieve the high yield purification purposes [27].

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Disclosure

The authors report no conflict of interest in this study.

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Table 1. The results of buffer condition changes in the chromatography column G25. Each sample was tested three times with each buffer and the average results were recorded.

Sample	buffer		average results	
	pH	Conductivity ms/cm	OD of protein	total protein mg/ml
1	5/06	3/82	0/205	0/07
2	5/02	3/05	0/184	0/05
3	/923	3/8	0/151	0/02
4	5	3/93	0/205	0/07
5	5/1	3/47	0/221	0/08
6	4/84	3/39	0/210	0/07
7	5/11	4/17	0/155	0/03
8	4/87	3/87	0/178	0/05
9	4/78	4/1	0/236	0/09
10	5/2	4/2	0/167	0/04
11	5/17	3/8	0/170	0/04
12	4/87	4/32	0/184	0/05
13	4/82	4/13	0/164	0/04
14	/884	4/01	0/192	0/06
15	4/87	3/89	0/163	0/04

Table 2. The results of buffer condition changes in the chromatography column ION exchange. Each sample was tested three times with each buffer and the average results were recorded.

sample	buffer		average results	
	pH	Conductivity ms/cm	OD of protein	Total protein mg/ml
1	5/18	30/1	0/349	0/33
2	4/8	30/3	0/270	0/25
3	4/68	32/1	0/158	0/16
4	4/99	30/5	0/222	0/22
5	4/98	32	0/215	0/21
6	4/75	31/3	0/165	0/17
7	4/96	33/9	0/165	0/17
8	4/91	35/3	0/262	0/24
9	5/07	32/3	0/272	0/25
10	5	33/8	0/278	0/26
11	5/16	32/5	0/216	0/21
12	4/87	33/8	0/210	0/2
13	4/95	34/2	0/193	0/19
14	4/87	32/1	0/191	0/19
15	4/95	34/2	0/158	0/16

Table 3. The results of buffer condition changes in the chromatography column G200. Each sample was tested three times with each buffer and the average results were recorded.

sample	buffer		average results	
	pH	Conductivity ms/cm	OD of protein	Total protein mg/ml
1	7/1	15/45	0/488	0/51
2	7	15	0/501	0/68
3	7/07	15/07	0/216	0/26
4	7/15	15/31	0/249	0/35
5	7/14	15/28	0/264	0/34
6	6/97	15/1	0/228	0/29
7	7/03	16/35	0/205	0/21

8	7/06	16/44	0/235	0/34
9	7/11	15/2	0/268	0/36
10	5/7	16/2	0/35	0/41
11	7/1	17/44	0/3	0/39
12	7/02	16/11	0/245	0/34
13	7/11	15/92	0/27	0/36
14	7/02	16/11	0/225	0/29
15	7/11	15/92	0/295	0/38

Table 4. The results of CZE.

isoform	1	2	3	4	5	6	7	8
Acceptable content	0-15	0-15	11-20	10-35	15-40	10-35	5-25	0-15
standard	0/4	1/9	7/7	17/8	31	24/3	12/3	4/1
1	0/9	3/1	6/7	15/8	32/3	12/5	13/2	3/4
2	0/7	2	5/8	18/6	27/7	27/6	16	1/2
3	2/3	11/4	22/4**	23/3	18/4	12/6	6/4	2/7
4	1/08	6/5	18/36	26/21	23	15/03	7/39	2/31
5	0/5	4/35	17/21	20/08	23/24	18/41	14/31	1/9
6	1/8	2/7	6/9	14/17	24/66	23/7	22/87	3/2
7	1/2	2/95	9/8	15/9	26/19	17/1	19/26	7/6
8	3/34	10/3	16/4	17/30	23/18	16/24	8/14	5/1
9	2/07	6/13	12/98	11/18	33/67	22/74	9/17	2/06
10	1/9	4/6	11/07	14/81	30/43	16/24	16/35	4/6
11	0/6	5/61	9/8	15/34	28/79	21/65	14/31	3/9
12	1/1	3/1	14/7	19/11	22/35	17/25	18/2	4/19
13	2/01	2/41	13/11	17/61	19/23	20/32	16/41	8/9
14	1/67	5/82	9/3	11/41	21/3	24/43	19/25	6/82
15	0/3	4/26	11/33	21/15	18/41	27/72	14/53	2/3

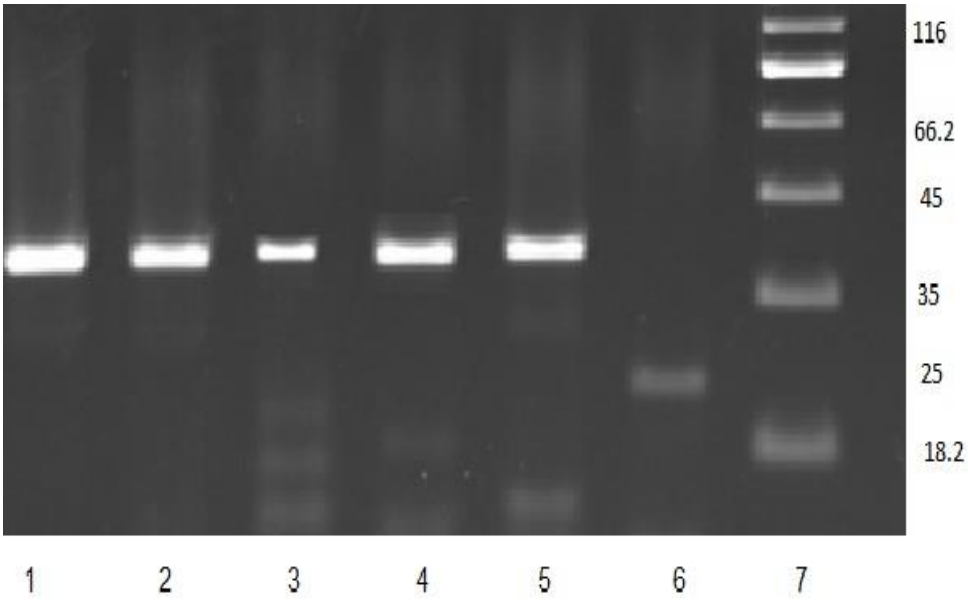

Figure 1- SDS PAGE; 7: marker (14/4-116 kda); 1, 2, 3, 4: samples; 5: positive control; 6: negative control.

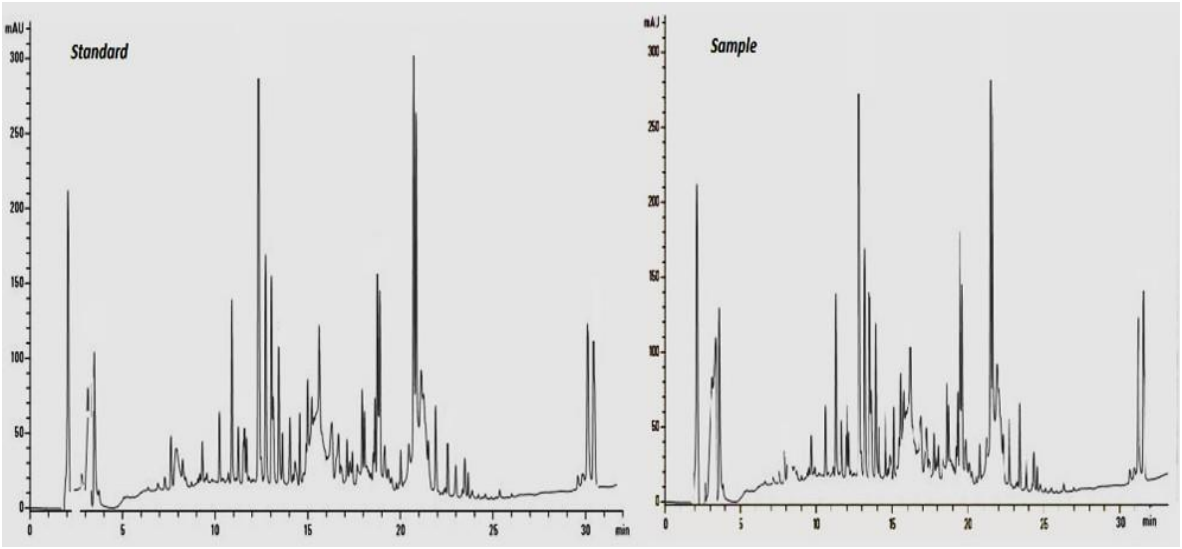

Figure 2- Comparison of erythropoietin peptide map. Left: Standard sample chromatogram; Right: sample chromatogram.

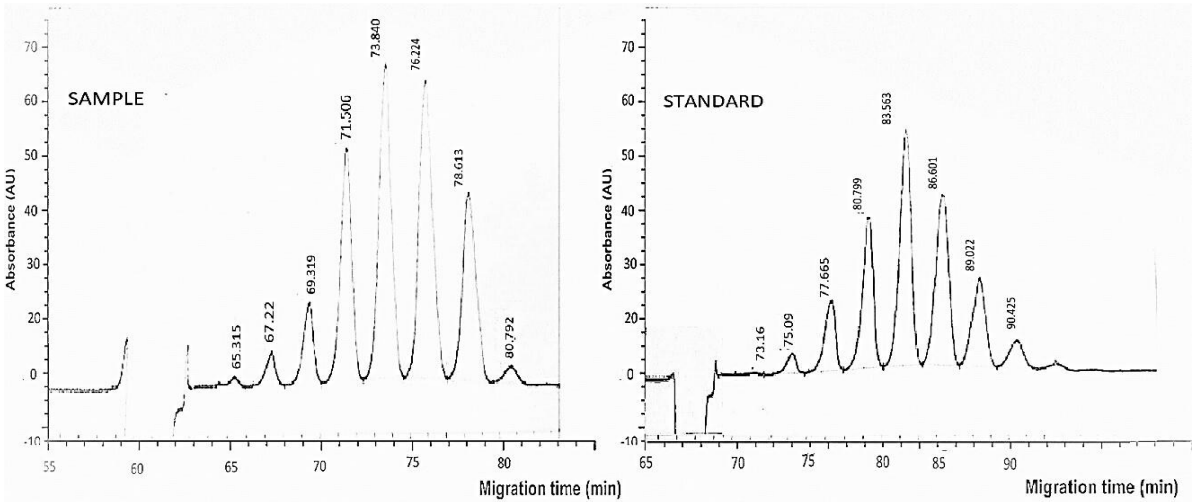

Figure 3- The CZE graph. Distribution of isoform of the sample (left) compared with the standard pattern (right).