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Effects of industrial ultrafiltration process condition on the purity and structure of recombinant erythropoietin

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Abstract

The ultrafiltration process is used widely to eliminate impurities during the downstream processing from recombinant proteins. Two ultrafiltration buffers (citrate and phosphate) are commonly used in the industrial production of recombinant erythropoietin. The aim of this study was to investigate the effect of these two buffers on the purity and structure of purified recombinant erythropoietin. In this study, seven harvests were purified by gel filtration and ion-exchange chromatography. Then, the ultrafiltration process was performed. So, half volume of each sample was washed with phosphate buffer and the rest with citrate buffer under different conditions. Purified protein content was measured using the HPLC method. The progeny, CHO protein contamination, ds-DNA impurity, and endotoxin level tests were performed. Finally, the structure was investigated by a capillary zone electrophoresis method. The results showed that the amount of obtained proteins were 0.51, 0.46, 0.38, 0.27, 0.39, 0.29, 0.38 mg/ml, and 0.46, 0.38, 0.35, 0.23, 0.36, 0.25, 0.34 mg/ml by washing with phosphate and citrate buffers, respectively. Qualitative and structural tests were in acceptable range for all proteins. The results of the study demonstrated that buffer conditions can have a noticeable effect on the efficacy of ultrafiltration process.

Key words: recombinant erythropoietin, ultrafiltration, Purification, impurity removal, Capillary zone electrophoresis

Introduction

Human Erythropoietin (EPO) is a glycoprotein hormone that is responsible for regulating red blood cell count (Yang et al., 2017). This protein is produced more by the kidneys and slightly by the liver. It stimulates the production of red blood cells and reduces the oxygen supply to the tissues (Papanikolaou and Pantopoulos, 2017; Wüstenberg et al., 2011). Erythropoietin acts as a factor in the erythrocyte precursors to differentiation in the erythroid cells (Wu et al., 2016). EPO production is reduced in the body in some pathological conditions such as renal parenchymal damage leading to anemia with varying degrees (Camaschella et al., 2016). this drug formulation is based on DNA (recombinant) technology (Zamanian et al., 2017). Recombinant erythropoietin is an important pharmaceutical for treatment of blood problems in children with chronic renal failure, malignancy, and cancer (Sankaran and Weiss, 2015; Moore and Bellomo, 2011).

During the production process, mammalian cell lines are used to make the glycosylated portion of the protein, which involved in the biological activity of the drug (Shahrokh et al., 2011). That leads to a lot of impurities in the culture medium (Butler and Spearman, 2014). The filtration process, especially ultrafiltration, plays a major role in purifying the drug (Machado et al., 2018). Two conventional buffers are used to perform the ultrafiltration process (Ashby et al., 2017; Maria et al., 2015). Phosphate and citrate buffers are commonly used by pharmaceutical companies (LaCava et al., 2016; Tanaka et al., 2016). Each of these buffers has disadvantages and advantages (Zbacnik et al., 2017).

The aim of the present study was to evaluate the effect of the buffers in industrial purification efficiency, as well as the possible advantages and disadvantages of these buffers on the purified protein structure.

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Materials and Methods

Sample collection method

For this study, recombinant erythropoietin protein was used as a model. Protein production was carried out through the culture of the CHO cell line containing dhfr (dihydrofolate reductase) plasmid in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, USA) containing human insulin as a supplement (Barnard et al., 2015). After growth and expansion, recovery stages, the supernatant was collected. After initial filtration with 0.2 μm membrane filter, initial chromatography (gel filtration and ion exchange) was performed to remove excess material from the harvest. Finally, the ultrafiltration system was introduced for final purification (Lipnizki, 2005).

In order to carry out the study, initially, the harvest was obtained from homogenized cell culture. Then, primary purification was done by gel filtration chromatography. Purposely, the sample was applied to a G25 column with Sephadex platform and washed with sodium acetate buffer. Subsequently, the eluted samples were homogeneous and applied to the Q-Sepharose ion-exchange chromatography column and washed in three steps with buffers of sodium acetate (pH 5), sodium acetate (pH 5) and sodium chloride. The obtained protein from this stage was introduced into the ultrafiltration. In each ultrafiltration process, 2.5 liters of sample was introduced into the ultrafiltration tank. In the first stage, the filtration process was performed for seven samples with citrate buffer under different pH and conductivity conditions, and then this process was investigated for seven other samples with phosphate buffer under different pH and conductivity conditions. In each step of this study, a peristaltic pump with a power of 10% and a constant flow rate was used to eliminate the influence of other factors on the efficiency. The amount of protein obtained in different buffering conditions was measured using spectrophotometry. Finally, the values of the obtained monomeric form of erythropoietin form (active pharmaceutical form) were measured from each filtration series by HPLC (Osman and Elbashir, 2017).

Qualitative study

In order to investigate the effect of buffers on the quality and purity of the recombinant protein, impurity determination tests were carried out including endotoxin level, pyrogenic in laboratory animals, presence of ds-DNA impurities, and the presence of CHO proteins (Görög, 2015). At the following stage, erythropoietin peptide map was determined by RP-HPLC. For this test, samples diluted with Tris 0.1 M to reach a concentration of 1 mg/ml. Then, 25 ml of the diluted sample centrifuged (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.

The capillary zone electrophoresis CZE test was performed to determine the effect of buffers on the glycosylation pattern of eluted protein (Zhang et al., 2015). Data obtained in this study were analyzed by T-test using SPSS software v. 19.

Results

The filtration process was performed according to the protocol of purification of recombinant Erythropoietin of WHO Technical Report Series, no.937, 2006 annex 4 used in the Pasteur Institute of Iran. The process was performed for samples with phosphate and citrate buffers with different pH and conductivity within the standard range. The results of the monomer form of eluted protein were determined by the HPLC method in different buffering conditions (Table 1).

In the following process, the impurity analysis for the samples obtained by filtration method was investigated by four methods. Initially, the presence of ds-DNA was detected by the Dig DNA marking & detection kit (Figure 1). The permitted range of ds-DNA impurities for the samples according to the protocol is ≤10 pg/dose. The results for both groups are shown in Table 2. In the next step, the endotoxin test was performed by chromogenic method using CAMBREX chromogenic LAL kit; the acceptable limits are ≤20 IU / 100000 IU EPO and the test results are recorded for both groups (Table 2). Then, the contamination amount of each sample with CHO cell line proteins was performed by using Cygnus method with CHO host cell protein kit. The authorized limit in the final material is less than 10 ppm in standard form. The above test results in the samples are shown in Table 2. Finally, a pyrogenic study was performed using BP method. In this stage, each sample was injected into New Zealand Rabbit at three stages. Measurement of animal fever rate was performed using the Ellab machine and the average of all three tests was recorded as the final result (Table 3). The peptide map of the samples was analyzed by the RP-HPLC method. The peptide maps obtained from the samples were similar to the standard sample pattern (Figure 2). At the next stage, CZE test was used to determine the effect of buffers on the glycosylated structure of protein. To prepare the sample for testing, the sample was initially desalinated and then electrophoresed with the standard sample (Figure 3). The distribution pattern of the samples' isoforms was compared with the standard and the results were recorded (Table 4). The obtained results showed that using phosphate buffers to purify the recombinant protein in the ultrafiltration process has significantly higher efficiencies than the citrate buffer.

Discussion and Conclusion

When the recombinant protein is placed under high pressure conditions, such as pH and conductivity changes, due to its sensitive physicochemical structures (Chang et al., 2013), it increases the likelihood of converting the monomeric structure of the recombinant protein into unacceptable forms such as dimer and aggregation (Kim et al., 2015; Alley et al., 2013). There is a chance to lose a lot of protein in the final purification of the ultrafiltration process (Ciceri et al., 2016; Rosa et al., 2010). On the other hand, this procedure is the main process for the removal of microbial, viral, endotoxin, ds-DNAs impurities, and CHO proteins from the harvest sample. Due to the optimistic results, it can be considered as a way to increase production efficacy of recombinant protein with being impacted by the removal of the above-mentioned impurities. In this study, we tried to investigate the common ultrafiltration buffers in industrial purification of recombinant erythropoietin, and the effect of buffers under different pH and conductivity conditions on the output of system. In order to investigate the effect of these changes on the rate of removal of impurities, quality control tests were performed per samples for various contaminations.

In a study by Ghosh et al. (2000), the effect of ultrafiltration system was investigated on isolation of lysozyme enzyme from chicken egg white. In this research, the ultrafiltration membrane was pre-treated with myoglobin. The results showed that the efficiency of the process had a 26% increase due to the effect of this material on the zeta potential of membrane (Ghosh and Cui, 2000). In the other study presented by Sen and colleagues in 2005, the effect of the ultrafiltration process on surfactant purification process was investigated. In this study, pressure and pH were studied as two factors of ultrafiltration. The results showed that by changing these two factors, the final concentration of surfactant changed (Sen and Swaminathan, 2005). In published research by Liu and colleagues in 2007, effective parameters in the ultrafiltration process were studied during the process of glucose oxidation in the bioreactor. In this research, temperature, pH, and buffer concentrations were investigated. The results showed that process efficiency increased up to 53% in optimal conditions. This suggests the effect of physicochemical factors on the ultrafiltration process (Liu and Cui, 2007).

The results of this study indicated that phosphate buffering system has better efficacy in the purification process than the citrate buffering system. Although the results showed that, in spite of buffering changes in both buffers, the impurities in the final sample were performed in acceptable range of above tests, and the isoform pattern and glycosylated structure of proteins did not alter. Future studies can be done by maintaining qualitative conditions on a wider scale and using software-based systems and artificial intelligence to examine the increase in efficiency (Charcosset, 2006).

Disclosure

The authors report no conflict of interest in this study

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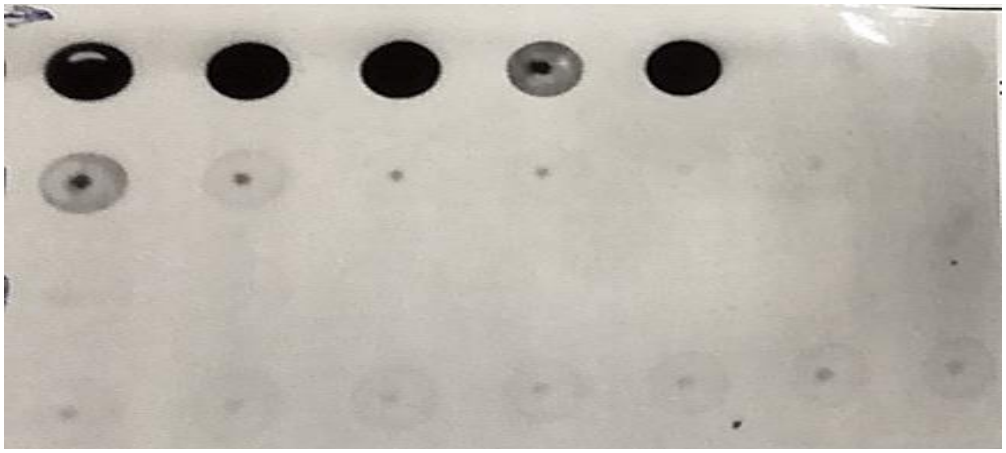

Figure 1 – Determination of the amount of dsDNA impurities: The samples and dilutions used in Figure are:
1- CHO DNA (100, 50, 25, 1, and 50 ng)
2- CHO DNA (500, 100, 50, 25, 10, and 5 ng)
3. Sample No. 1: EPO-API - 01
4. Sample number one + 25pg CHO DNA

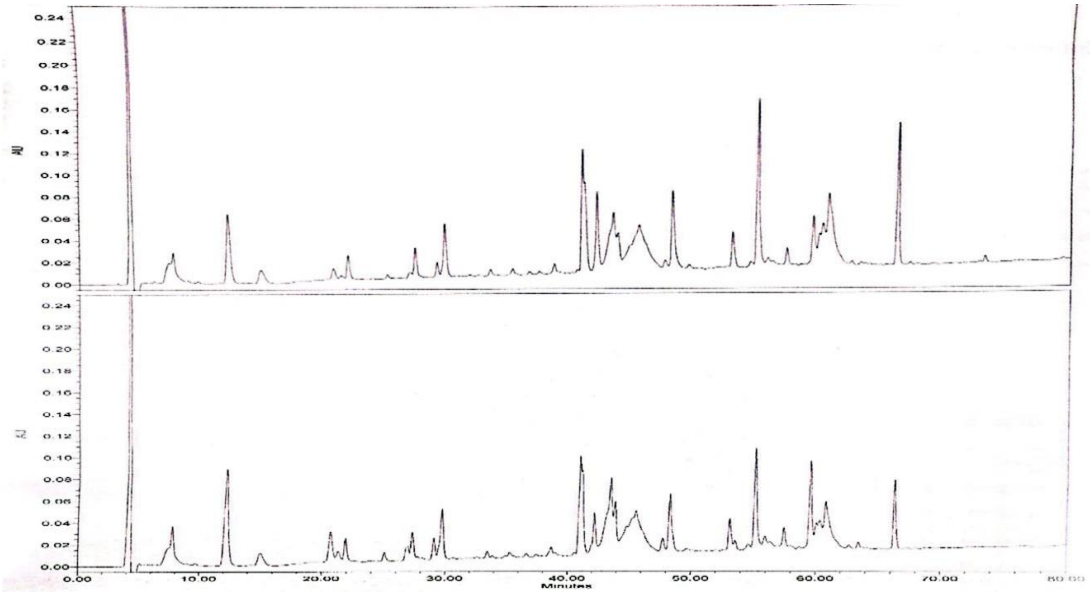

Figure 2– Comparison of erythropoietin peptide map .Up: Standard sample chromatogram ؛Down: sample chromatogram

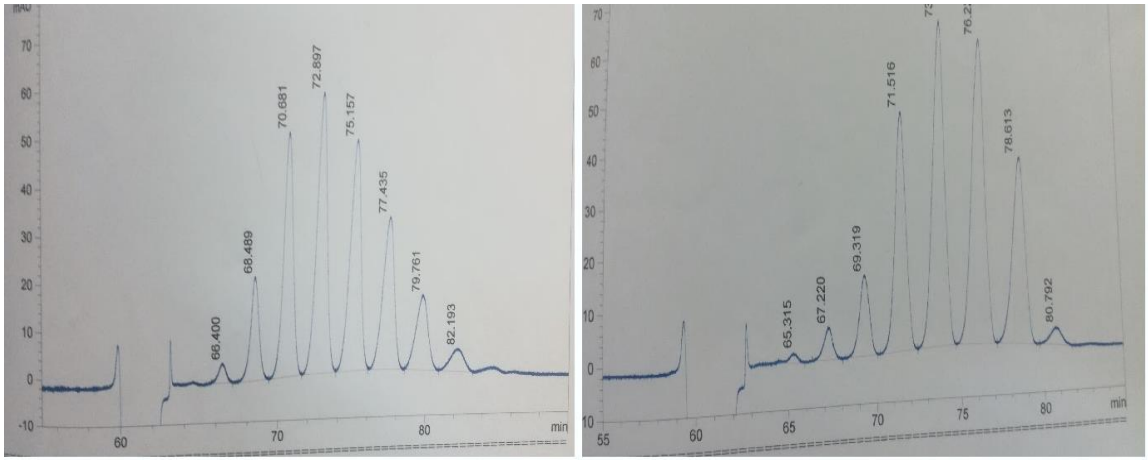

Figure 3 –CZE test: pattern isoform of sample (left), pattern isoform of standard (right)

Table 1. Results of ultrafiltration with phosphate and citrate buffer with different pH and conductivity

Ultrafiltration with phosphate buffer				
HARVEST no.	pH of buffer	conductivity	OD	Monomer concentration mg/ml
1	7.4	15.43	0.275	0.38
2	7.3	15.06	0.256	0.29
3	7.1	16.97	0.315	0.46
4	6.8	15.31	0.237	0.27
5	7	16.1	0.288	0.38
6	7.2	15.34	0.349	0.51
7	7.1	15.73	0.289	0.39
Ultrafiltration with citrate buffer				
1	6.8	13.8	0.214	0.33
2	6.7	14.1	0.223	0.25
3	6.8	14.4	0.207	0.38
4	6.9	13.5	0.241	0.23
5	6.7	12.7	0.218	0.35
6	7.0	13.6	0.233	0.46
7	6.7	13.9	0.251	0.36

Table 2. Result of impurity test include ds-DNA, endotoxin level, CHO cell line protein

	Harvest Ultrafiltration with phosphate buffer							Harvest Ultrafiltration with citrate buffer						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ds-DNA impurities	7	5	6	8	4	5	3	5	5	6	8	3	3	5
endotoxin test	0.1	0.1	0.3	0.1	0.5	0.3	0.1	0.1	0.6	0.1	0.2	0.4	0.1	0.5
CHO cell line	0.08	0.07	0.07	0.09	0.1	0.08	0.09	0.07	0.1	0.08	0.09	0.08	0.07	0.07

Table 3. Results of the difference in mean temperature before and after three times injection of eluted protein in the three New Zealand rabbits. The difference of more than 1.5 ℃ means that the protein is febrile.

Sample No.	Body temperaturebefore injection (Ti) in three rabbits (℃)			Body temperature after injection (Tm) in three rabbits (℃)			The average temperature difference before and after injection	result
1	39.4	38.8	30.0	39.4	38.9	39.1	≤ 1.5 ^{0}C	Accept
2	39.2	39.3	38.9	39.4	39.3	38.9	≤ 1.5 ^{0}C	Accept
3	39.5	38.5	39.2	39.6	38.9	39.4	≤ 1.5 ^{0}C	Accept
4	38.8	38.2	38.4	38.8	38.5	38.8	≤ 1.5 ^{0}C	Accept
5	38.6	38.8	39.0	39.0	39.1	39.1	≤ 1.5 ^{0}C	Accept
6	39.0	38.1	39.0	39.2	38.3	39.3	≤ 1.5 ^{0}C	Accept
7	39.2	39.1	38.4	39.2	39.2	38.6	≤ 1.5 ^{0}C	Accept
8	38.8	39.0	38.3	38.9	39.3	38.5	≤ 1.5 ^{0}C	Accept
9	39.0	39.3	39.2	39.0	39.5	39.2	≤ 1.5 ^{0}C	Accept
10	38.7	39.1	39.1	38.9	39.4	39.2	≤ 1.5 ^{0}C	Accept
11	38.3	39.0	39.0	38.7	39.0	39.3	≤ 1.5 ^{0}C	Accept
12	39.1	38.4	38.6	39.4	38.9	38.7	≤ 1.5 ^{0}C	Accept
13	39.1	38.4	38.2	39.4	38.7	38.4	≤ 1.5 ^{0}C	Accept
14	39.0	38.3	39.1	39.0	38.6	39.3	≤ 1.5 ^{0}C	Accept

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