Original Article

Optimization of ultrafiltration process for human recombinant erythropoietin purification

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ABSTRACT

Ultrafiltration plays an important role in purification process of recombinant proteins. In this study, the efficiency of the process was assessed by changing buffering condition. Initially, the harvests were purified by primary chromatographic methods. Then, the samples were applied to the ultrafiltration and washed with different pH and conductivity buffers. Proteins purity tests were done and the peptide map investigated by the RP-HPLC method. The research results showed that changes in buffering conditions can improve the efficacy of ultrafiltration and some physicochemical properties had a significant effects on the final resultsUltrafiltration plays an important role in purification process of recombinant proteins. In this study, the efficiency of the process was assessed by changing buffering condition. Initially, the harvests were purified by primary chromatographic methods. Then, the samples were applied to the ultrafiltration and washed with different pH and conductivity buffers. Proteins purity tests were done and the peptide map investigated by the RP-HPLC method. The research results showed that changes in buffering conditions can improve the efficacy of ultrafiltration and some physicochemical properties had a significant effects on the final results.

Keywords: Human Recombinant proteins, ultrafiltration, purification process, optimization.

Introduction

Production of recombinant therapeutics in recent years has become significant part of the pharmaceutical industry in the world because of increased demand of such products [1]. Therefore, there is a widespread attention to this kind of therapeutics among the respective companies. So because mammalian cell lines , based on the manufacturing point of view, are used to achieve the appropriate structure of these therapeutic proteins, the purification process of these drugs has a high complexity, is quite costly, and is always considered as a bottleneck in the economy [2].

Access this article online	
Website: www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Hamid Liryaei, Hooman Kaghazian, Mohammad Reza Akbari Eidgahi. Optimization of ultrafiltration process for human recombinant erythropoietin purification. J Adv Pharm Edu Res 2020;10(2):155-160. Source of Support: Nil, Conflict of Interest: None declared.

Isolation and purification of proteins during downstream processes is very costly and is considered as a bottleneck in the production of recombinant drugs. In fact, the cost of the downstream phase is 70 to 80% of the total cost of the production of the recombinant drug and other biomolecules [3].

Since one of the most significant indications of pharmaceutical standards for production of recombinant proteins is purity, researchers and pharmaceutical companies try to make the highest amount of high quality protein at the lowest possible cost [4]. In conventional industrial settings, some methods such as viral filtration, ultrafiltration, and diafiltration used to achieve this purpose.

In pharmaceutical industries, ultrafiltration is one of the most important purification methods which in addition to increasing efficiency it can facilitate the production process and the possibility of using a continuous process as well as maximizing the desired protein recovery, saving energy, and reducing production time [5]. Other benefits of ultrafiltration in pharmaceutical industries (enzymes, antibiotics) include removing bacteria and viruses, lowered operation pressure,

increase deficiency, reduced need for chemicals, and lowered operating costs [6].

Ultrafiltration is a membrane-based technology based on semi-permeable membranes to separate fractions of solutions containing beneficial and non-toxic compounds. Ultrafiltration, which is widely used in the pharmaceutical industry, can be described as an important technique in the process of separation and concentration of proteins [7]. However, the efficacy of this method in the purification of recombinant drugs is highly dependent on various factors such as pressure, pH, temperature, and time of the process. Consequently, the efficiency of the filtration process can be changed by changing the factors affecting ultrafiltration, by evaluating the effective factors involved in ultrafiltration [8]. The purpose of this study was to determine the optimal conditions of ultrafiltration for purification of recombinant erythropoietin as a model [9].

Materials and Methods

• Sample collection

Recombinant erythropoietin protein was selected as a model of pharmaceutical proteins [10]. It was obtained by cultivating CHO cell line containing dhfr$^-$ plasmid. The cell line was cultured in a selective DMEM medium, and after collecting the harvest and performing initial chromatography, ultrafiltration process was investigated [11].

• Method of performing the process

This study was carry out according to the protocol of purification of recombinant Erythropoietin of WHO Technical Report Series, no.937,2006 annex 4. For the ultrafiltration process, proteins that were initially purified by chromatography methods were used. At the beginning of each series, ultrafiltration system was rinsed with WFI. The pressure of the peristaltic pump was in the same conditions at the start of the process for all samples. Then, the Proflux M2 ultrafiltration system was balanced with phosphate buffer. Afterwards, based on the protocols of recombinant erythropoietin produced by Pasteur Institute of Iran, we applied samples into ultrafiltration vessel and washed it twice with phosphate buffer to carry out the ultrafiltration process.

Phosphate buffer with different pH and conductivity were used to study these influencing factors [12]. Each buffer was examined three times with the sample and examined for the mentioned criteria, then the average results of each buffer were recorded. The duration of ultrafiltration in the experiments was 5 to 10 minutes, and the pressure difference was 0.5 bar.

Because erythropoietin monomer is used as an active form of the pharmaceutical, samples obtained from each filtration method were investigated with HPLC to investigate the effect of changes in influencing factors on the system's quantitative efficiency [13]. Then ODs of suspensions were examined by spectrophotometry at absorbance 280 nm. In order to evaluate the purity of the recombinant protein, endotoxin LAL test, Pyrogenic test in laboratory animals, dsDNA impurity test, and CHO contamination by Cygnus kit was performed [14].

Finally, 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 centrifuge with 5000 rpm for 5 minutes. After that, 0.2 ml of DI water was added and the centrifuge was done again. After desalting, 5 µg of trypsin 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 together. Data obtained were analyzed using Pearson correlation test and SPSS software 19v from IBM.

Results

After performing the process with different pHs and conductivities of buffer, HPLC method was used to check the yield of obtained monomer form of protein. The results of HPLC were as follows. (Table 1)

In the next stage of the study, three tests were carried out. The active pharmaceutical ingredient is licensed to enter the formulation stage when the results of these tests are within the acceptable limits of the standard. At first the double-stranded DNA impurity test was performed. To perform this test, a Dig DNA marking & detection kit with standard DNA was used. The acceptable range is ≤10pg/dose. The results showed that in all samples, the impurity level was within the acceptable range of the standard protocol. In the next step, endotoxin test was performed by using CAMBREX chromogenic LAL kit, the acceptable range is ≤20 IU / 100000 IU EPO. The results showed that, in each sample, the level of endotoxin is within acceptable limits for use in the clinic. In the following the amount of contamination in the samples with CHO cell line proteins was examined using a CHO host cell protein kit (Figure 1). It is worth noting that one of the most important indicators of recombinant protein purity during the production process is the determination of the amount of host cell proteins in the obtained samples. The standard limit for this impurity in the final material is less than 10ppm.The

Qualitative test results are presented in the table below (Table 2). All samples were within acceptable limits.In the next stage, Pyrogenic study was performed by using laboratory animals. At each stage, three New Zealand rabbits were selected for each sample. Body temperature of the animals was measured by using Automated Ellab. In this test, if the temperature difference before and after three times injection of sample was more than 1.5 ° C, the sample was considered as an unacceptable sample. The results showed that the pyrogenic test was negative for all specimens. (Table 3)

At the end of the study, the peptide map of the samples were analyzed by the RP-HPLC method. The peptide maps obtained

from the samples were similar to the standard sample pattern. (Figure 2)

Statistical analysis

The statistical analysis showed that there is a significant difference between each series of experiments ($P<0.0001$), which indicates the effect of changes in buffering conditions on the performance of the ultrafiltration process. (Figure 3)

Discussion and Conclusion

The recombinant erythropoietin, like all recombinant proteins, has an unstable structure, and this structure is susceptible to physical and chemical agents.

On the other hand, the most important purification process for the removal of microbial, viral, endotoxin, dsDNA impurity and CHO proteins contamination from collected harvest is ultrafiltration [15]. Due to the fact that during this process, the protein is washed out by buffers with pHs and conductivities irrelevant to the erythropoietin's optimal conditions, thus increasing the possibility of converting the monomer structure into dimers and aggregation form. Optimizing the filtration process without affecting the erythropoietin structure can be considered as a way to increase the efficiency of the production of the recombinant protein.

In this study, we tried to investigate the effect of the filtration process on the efficiency of the production of recombinant erythropoietin. To investigate the effect of these changes on the process output and the structure and removal of impurities, the filtration process was carried out under different conditions of buffering.

It should be noted that, although the ultrafiltration is a significant part of the purification process, little research has been done to improve this process.

One of the studies in the field of software development to increase the quality of production led to Mutara EMI Filter Selection Simulator software. It simulates the best functionality of filters based on the information of databases. In another study, ROSA system design software was developed, which is a modeling system for evaluation of the factors influencing production process. BRUKER Company has developed a system, PAT, for optimization of therapeutics analysis [16]. This system mostly optimizes FT-IR, FT-NIR, and Raman spectroscopy. SARTOFLOW Slice 200 Benchtop System is one of the advancements aiming concentration, diafilteration, ultrafiltration, and microfiltration during purification [17]. It has been designed to optimize purification and concentration of proteins and uses a wide spectrum of filter (0.2 micrometer to 0.14 nanometer). One of the advantages of SARTOFLOW system is that it gives the optimum condition for various environmental conditions. For example, 20 milliliters of a desired product can be subject to ultrafiltration, and a considerable change in the quality and purity of the final product will be achieved [18]. There are three automatic program designed for ultrafiltration, diafiltration, and microfiltration. The following table shows example data of SARTOFLOW for optimization of ultrafiltration of protein products [19].

During various experiments, data are transferred to computers, and the software presents the information of different experimental conditions as graphs in Excel format. Finally, it directs the operator to the best possible condition by indicating the exact value of variables. Nonetheless, it is only applicable for experimental, small-scale, and pilot studies.

There are several reasons for more thorough studies on the factors influencing the output of erythropoietin purification, i.e. in the previous studies, few numbers of factors have been evaluated, and modeling software applications were used only for one filtration method. Furthermore, there is no comprehensive software to simulate the industrial production of recombinant proteins.

Importing such technologies has a great financial burden, pharmaceutical filtration methods used are considered as a bottleneck that lowers the production speed, and these filtration methods are not cost-benefit [20]. Therefore, the is demanding need for the development of software applications that are capable of optimizing the whole process of industrial production of recombinant pharmaceuticals to achieve the highest possible output.

The statistical analysis showed that buffers number 9 and 10 had the highest yield, while buffers number 5 and 6 had the lowest output. Although the values obtained are close together, but on a higher scale (especially industry), these changes can be very significant. These results represent that the minimal variations in the various factors of the filtration process, such as flow and temperature, pH and conductivity, change the process efficiency. In addition, the results showed that although buffers with different pH and conductivity were used to examine the ultrafiltration process, the amount of impurities in the final sample were within the acceptable range [21].

Therefore, in order to increase the efficiency of the ultrafiltration process, it is possible to use software-based systems, to simulate the filtration process in different conditions. This will probably increase the efficiency while maintaining the desired structure.

Funding

This research was financially supported by Semnan University of Medical Sciences (SUMS) grant.

Conflicts of Interests

There is no conflict of interest.

Financial Disclosure

No financial interests related to the material of this manuscript have been declared.

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Table 1. Result of HPLC: polishing stage with different PH and conductivity of phosphate buffers. Each sample was examined three times with each buffer. The average results were recorded as follows.

phosphate buffer	pH	Conductivity	Temperature Of process	average results	
				OD	Monomer concentration mg/ml
1	7.1	15.45	22.2	0.257	0.33
2	7.4	16.44	22.1	0.245	0.29
3	7.1	15.07	21.7	0.319	0.49
4	6.9	15.14	22.3	0.289	0.37
5	7.4	15.28	22.6	0.225	0.28
6	7.5	15.21	22.2	0.214	0.26
7	7.3	16.97	22.5	0.249	0.31
8	7.2	16.11	22	0.269	0.35
9	6.8	15.01	22.3	0.533	0.68
10	6.9	15.12	22	0.488	0.51
11	7.1	15.57	22.2	0.273	0.36

Table 2- The results of Qualitative tests of sample eluted by ultrafiltration method.

sample	ds- DNA pg/dose	Endotoxin level per IU/100000 IU EPO	CHO host cell protein microgram
1	≤5	0.1	0.09
2	≤4	0.4	0.09
3	≤5	0.3	0.1
4	≤8	0.6	0.07
5	≤6	0.1	0.09
6	≤5	0.1	0.08
7	≤3	0.2	0.1
8	≤5	0.4	0.1
9	≤5	0.1	0.08
10	≤4	0.3	0.08
11	≤6	0.1	0.09

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

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

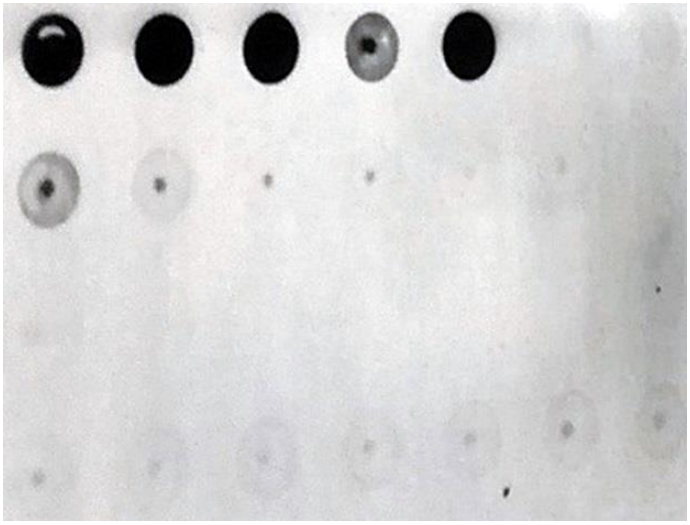

Figure 1 - Determine the amount of dsDNA impurities: The samples and dilutions used in Figure 1 are:
1- CHO DNA (100, 50, 25, 1, 50) ng
2- CHO DNA (500, 100, 50, 25, 10, 5) ng
3. Sample No. 1: EPO-API - 01
4. Sample number one + 25pg CHO DNA

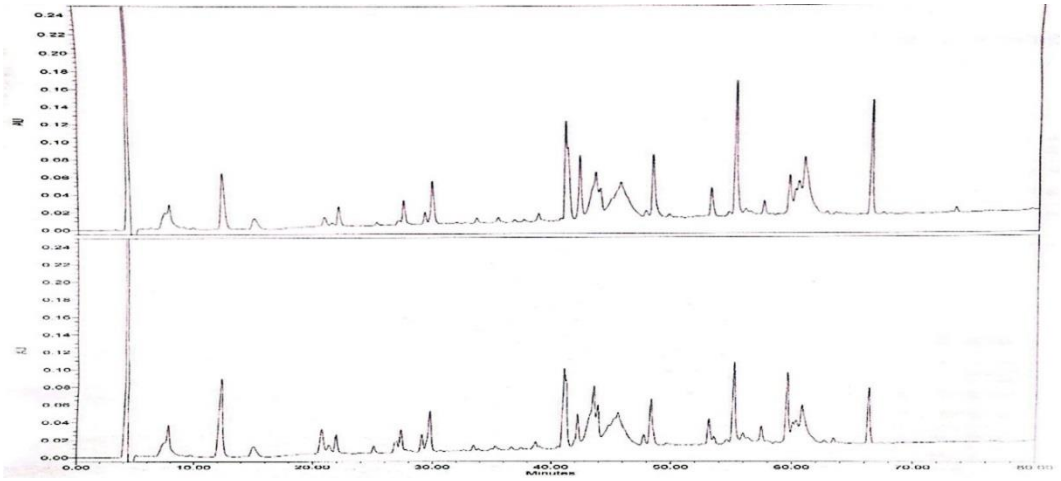

Figure 2– Comparison of erythropoietin peptide map .Up: Standard sample chromatogram ؛Down: sample chromatogram

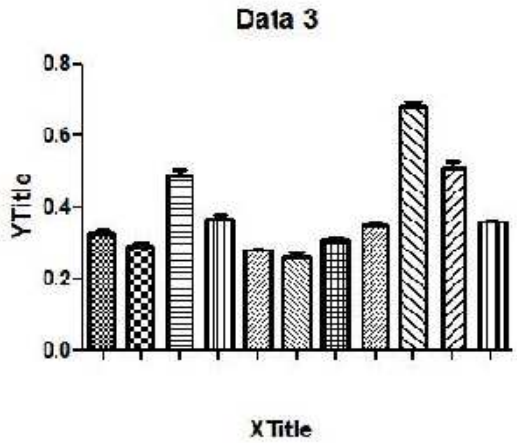

Figure 3- Statistical survey. The X column shows the number of buffers and the Y column represents the amount of protein obtained.