Original Article

Investigation of purification process stresses on erythropoietin peptide mapping profile

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Abstract

Background: Full compliance of recombinant protein peptide mapping chromatogram with the standard reference material, is one of the most basic quality control tests of biopharmaceuticals. Changing a single amino acid substitution or side chain diversity for a given peptide changes protein hydrophobicity and causes peak shape or retention time alteration in a peptide mapping assay. In this work, the effect of different stresses during the recombinant erythropoietin (EPO) purification process, including pH 4, pH 5, and room temperature were checked on product peptide mapping results.

Materials and Methods: Cell culture harvest was purified under stress by different chromatographic techniques consisting of gel filtration, anionic ion exchange, concentration by ultrafiltration, and high resolution size exclusion chromatography. To induce more pH stresses, the purified EPO was exposed to pH stress 4 and 5 by exchanging buffer by a 10 KDa dialysis sac overnight. The effects of temperature and partial deglycosylation (acid hydrolysis) on purified EPO were also studied by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and peptide mapping analysis. Removal of sialic acid by mild hydrolysis was performed by exposure to two molar acetic acid at 80°C for 3 h.

Results: No significant effect was observed between intact and stressed erythropoietin peptide mapping profiles and SDS-PAGE results. To validate the sensibility of the technique, erythropoietin was partially acid hydrolyzed and significant changes in the chromatographic peptide map of the intact form and a reduction on its molecular weight were detected, which indicates some partial deglycosylation.

Conclusions: Purification process does not alter the peptide mapping profile and purification process stresses are not the cause of peptide mapping noncompliance.

Key Words: Erythropoietin, peptide mapping, purification stresses

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INTRODUCTION

Recombinant biotherapeutics have growing markets[1,2] and complex structure as compared with chemotherapeutics,[3,4] which are more complex to analyze and thus need powerful analytical approaches such as chromatographic (high-pH anion-exchange chromatography, hydrophilic interaction chromatography, and gas chromatography) and electrophoretic techniques (isoelectric

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focusing, sodium dodecyl sulphate-polyacrylamide gel electrophoresis [SDS-PAGE], native PAGE and 2D electrophoresis [2D-PAGE], capillary iso-electrofocusing [CE], and microfluidics capillary iso-electrofocusing, fluorophore-assisted carbohydrate electrophoresis and sarcosyl-PAGE), mass spectrometry, and spectroscopic approaches.[5-9] Post-translational modifications such as glycosylation in recombinant erythropoietin at its glycan side chains (terminal ends) are capped with sialic acid, thus causes macro- and microheterogeneity and thereby imposing much complexity in protein structure.[10-13] Also different stresses on production process, for example, pH shift, temperature and pressure changes, presence of salts, metal ions and surfactants, foaming, pressure, shaking and shearing, absorption to surfaces, and protein concentration could induce physical conformational alterations (aggregation, precipitation, denaturation, and adsorption to surfaces) or chemical degradation, namely, inappropriate glycosylation, protein misfolding, deamidation, oxidation, cysteine β-elimination, fragmentation of disulfide bonds, crosslinking exchange; hydrolysis of backbone peptide and hydrolysis by proteases, trans-amidation, and chemically triggered nonspecific crosslinking, may affect biological activity, immunogenicity, stability, and other quality profiles of the products.[1,10,14-23]

Several tests and assays are necessary to ensure that the product meets quality requirements. Peptide mapping, which is used to identify primary structure (the amino acid sequence) and its derivatives such as glycosylation, oxidation, deamidation, or degradation when combined with other methods such as mass spectrometry, is one of the most important analytical tools required to perform in order to release batches of recombinant therapeutic proteins[20,22,24,25] besides other quality control requirements. This method uses the advantages of significant retention time differences and changing the hydrophobicity of a protein by changing a single amino acid substitution or side chain diversity for a given peptide.[26-28] Process variations usually affect the quality of the products, which can be detected by this method.[24] In brief, complexity and sensitivity of protein structure necessitate producers to check all structural quality aspects of their products and the effect of different production parameters on product heterogeneity and stability. Because most of the mentioned quality tests such as peptide mapping need a pure sample to be analyzed. However, the effects of purification process on products are important to be evaluated. In this way, the observed variation in obtained results is a response of the effect of process parameters, which is not an error but it could be due to poor sample treatments.

In this work, we have studied the effect of purification process stresses caused on recombinant human erythropoietin peptide mapping. Recombinant human erythropoietin (rhEPO) is a glycosylated protein, which has been used as a biotherapeutic agent since 1988 and prescribed for the treatment of anemia, especially anemia caused by renal failure, cancer, and HIV infection.[29,30] EPO is synthesized mainly in the kidney and stimulates erythropoiesis and red cell production. It consists of 165 amino acids being glycosylated with 34 KDa molecular weight. It contains two disulfide bonds, 40% of its structure is composed of three N-linked sites at asparagines residues (Asn24, Asn38, Asn83) and one O-linked oligosaccharide chain at serine residue.[31-35] The structures of the intact sialylated carbohydrate chains of rhEPO expressed in Chinese Hamster Ovary (CHO) cells including its primary structures, number, and the composition of branched antennary and sialylated N- and O-type oligosaccharides have been fully studied.[9,36] All related researches indicate that the sialic acid-containing carbohydrate content of rhEPO is directly proportional to the serum half-life and *in vivo* bioactivity.[10,35,37,38] Glycosylation is essential in maintaining the stability of proteins against pH denaturation,[39] tryptophan oxidation,[40] thermal denaturation,[10,41,42] aggregation,[43] and proteases degradation.[10,44] Different enzymes including proteases family especially exoproteases and glycosidases and temperature[10,35,45] or amino acid changes by environmental conditions[10,26] cause alteration in the EPO structure and thus deviation in peptide mapping profile. Particular glycosidase enzymes exist in mammalian cell cultures such as sialidase, beta-galactosidase, beta-hexoaminidase, manosidase, and fucosidase, which each of them remove certain sites from protein poly peptide backbone,[46,47] resulting in product heterogeneity and decreasing biological activity. In some production, some deviations in peptide mapping chromatogram profile are seen, which results to batch rejection. Hence finding the reasons of these deviations is an important point in quality risk assessment analysis. In this article, we report the exposure of recombinant alfa-erythropoietin active pharmaceutical ingredient (EPO API) to susceptible stresses during the purification process (different pHs and room temperature) and compare the peptide mapping chromatograms with intact protein to study the stability of recombinant erythropoietin by chromatographic peptide mapping against these stresses. We have also studied the effect of partial deglycosylation on peptide mapping of erythropoietin's profile to demonstrate the sensitivity of reversed phase chromatographic techniques.

MATERIALS AND METHODS

Recombinant erythropoietin purification process was performed on the harvest of Chinese hamster ovary (CHO) cells (dihydrofolate reductase or DHFR) producing rhEPO. The cell culture process was explained in a previous work.[48] Fifty milliliters of CHO cell culture harvest was applied, to gel filtration chromatography XK 26/40 column (GE Healthcare, Uppsala, Sweden,+46-18) packed with 175 mL Sephadex G25 media (GE Healthcare, Uppsala, Sweden, +46-18) pre-equilibrated with sodium acetate pH 5 buffer at 3.5 mL/min flow rate by a semi-preparative high performance liquid chromatography (HPLC) system (Waters, Milford, USA, +1-508). The eluted protein was applied to a XK50/20 column (GE Healthcare, Uppsala, Sweden, +46-18) packed with 60 mL anion exchange chromatography Q-Sepharose fast flow media (GE Healthcare, Uppsala, Sweden, +46-18) pre-equilibrated with above-mentioned buffer at 19.6 mL/min flow rate. Sodium acetate buffer pH 4 was used as washing step and 300 mM NaCl solution as elution step. The intermediate product from this step was concentrated using a 15 mL centrifugal tube with a 10 KDa filter Amicon-Ultra (Millipore, Billerica, USA, +1-978), then was applied at 2.65 mL/min flow rate to a XK 26/70 column (GE Healthcare, Uppsala, Sweden, +46-18) packed with 345 mL of high resolution gel filtration chromatography Superde × 200 prep grade media (GE Healthcare, Uppsala, Sweden, +46-18) pre-equilibrated with phosphate buffer 20 mM pH 7.

For removing sialic acid by mild hydrolysis, 1 mL of recombinant erythropoietin as an active pharmaceutical ingredient form (EPO API, Pasteur Institute of Iran, Iran, +98-26) was exposed to 2 molar acetic acid at 80°C for 3 h.[49] After incubation, 5 mL of phosphate buffer was added to the hydrolyzed mixture so as to dilute acid and the buffer exchange was performed with phosphate buffered saline using a 10 KDa dialysis bag at 4°C overnight. Dialysis bag was prepared by heating in a 2% (W/V) sodium bicarbonate, 1 mM EDTA, pH 8.0 for 10 min, washing with deionized water, again heating for 10 min with 1 mM EDTA, pH 8.0, and finally washing with deionized water.

Peptide mapping tests were performed according to British pharmacopeia (BP):[50] Intact EPO and test samples were desalted by tris–acetate buffer pH 8.5 using 0.5 mL Centrifugal Filters 10 KDa Amicon-Ultra (Millipore, Billerica, USA, +1-978). Protease digestion was accomplished by adding 1 mg/mL trypsin (Promega, Madison, USA, +1-608) at a substrate-to-enzyme ratio of 50:1 (w/w). The solution was mixed and incubated at 37°C for 18 h. The digestion was quenched by storing the sample at -70°C. A reverse phase chromatography column C4 (Waters, Milford, USA, +1-508) connected to an HPLC system (Waters, Milford, USA, +1-508), a gradient mobile phases of A (0.06% trifluroacetic acid, TFA) and B (100 mL of 0.6% TFA in 900 mL acetonitrile) was employed and 214 nm wave length was used for peaks detection.

SDS-PAGE and size exclusion HPLC methods were used for purity analysis. Size exclusion HPLC was performed according to British pharmacopeia (BP)[50] using a TSK gel G3000SW 7.5 mm ID × 60 cm L column (TOSOH, Tokyo, Japan). Reduced SDS-PAGE (12%) was performed according to standard procedure,[51] prior to SDS-PAGE, the sample was desalted by 10 KDa Amicon-Ultra (Millipore, Billerica, USA, +1-978). Marker No. 26610 (Termo Fisher Scientific, Waltham, USA, +1-781) and PM30-500 (DNA Gdansk, Gdansk, Poland, +48-58) was used as molecular weight ladders.

RESULTS

All the mentioned purification steps were performed at a temperature range of 6–8°C by circulating cold water through column jackets but at the end of each step, the intermediate products of each purification steps were kept at room temperature for 3 h to reach a range of 12–18°C (the worse experienced uncontrolled temperature range during real production process). The summary of erythropoietin purification process is shown in Table 1.

First gel filtration chromatography step was used to decrease the conductivity of the sample before applying to ion exchange column and no purification fold increase was expected. In the anionic exchange chromatography step, some contaminations and also

Table 1: Summary of erythropoietin purification process

Step	Volume (mL)	Total protein concentration (μg/mL)	Purity (%)	Total protein (mg)	Total EPO (mg)	Purification fold	Recovery (%)
Cell culture harvest	50	60	45.00[a]	3.00	1.35	–	100.00
Gel filtration (buffer exchange)	52	55	46.00[a]	2.86	1.32	1.02	97.45
Ion exchange chromatography	12	100	87.40[a]	1.20	1.05	1.94	77.69
High resolution gel filtration	38	24	100[b]	0.91	0.91	2.22	67.56

[a]By SDS-PAGE, [b]By size exclusion HPLC

deglycosylated erythropoietin was eliminated using a wash step by decreasing pH of mobile phase to pH 4. In this step, the basic form of erythropoietin, which has less sialic acid and some other contaminants was eliminated. The column was washed with this buffer continuously for an hour to simulate real production process stress exposure. The purified fraction was eluted from anion exchange column by stepwise increasing of the mobile phase conductivity using a 300 mM sodium chloride solution. The size exclusion chromatography was performed as polishing step. Low sample application volume (2%–4% of column packed bed volume) is necessary to achieve high resolution fractionation, thus a concentration step was used to decrease the volume of sample to 7 mL and finally a highly purified erythropoietin was obtained. Figure 1 shows the purity test results of each step. The peptide mapping analysis was performed to the purified erythropoietin under stress and any noncompliance was detected comparing the standard EPO peptide mapping profile.

To study the effect of mentioned stresses on erythropoietin structure itself, the purified EPO was exposed again to pH stress by exchanging phosphate buffer with the above-mentioned buffers (pH 4 and 5) through 10 KDa dialysis bag overnight. The effect of temperature (room

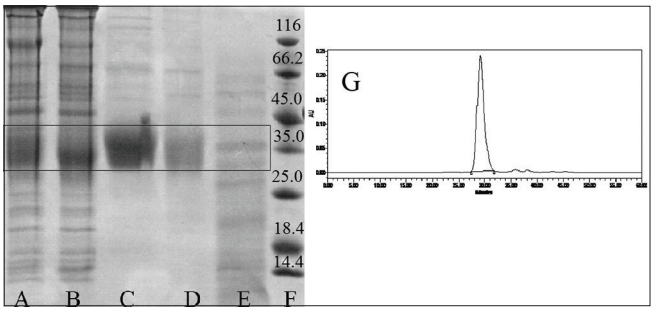

Figure 1: Purity test of purification steps intermediates. Rectangle region shows erythropoietin band. Lane A: Cell culture harvest, Lane B: Gel filtration buffer exchange; Lanes C and D; Anion exchange chromatography products 1 and two times diluted; Lane E: Anion exchange chromatography wash pH 4 fraction; Lane F: Molecular weight ladder; G: Size exclusion HPLC chromatogram

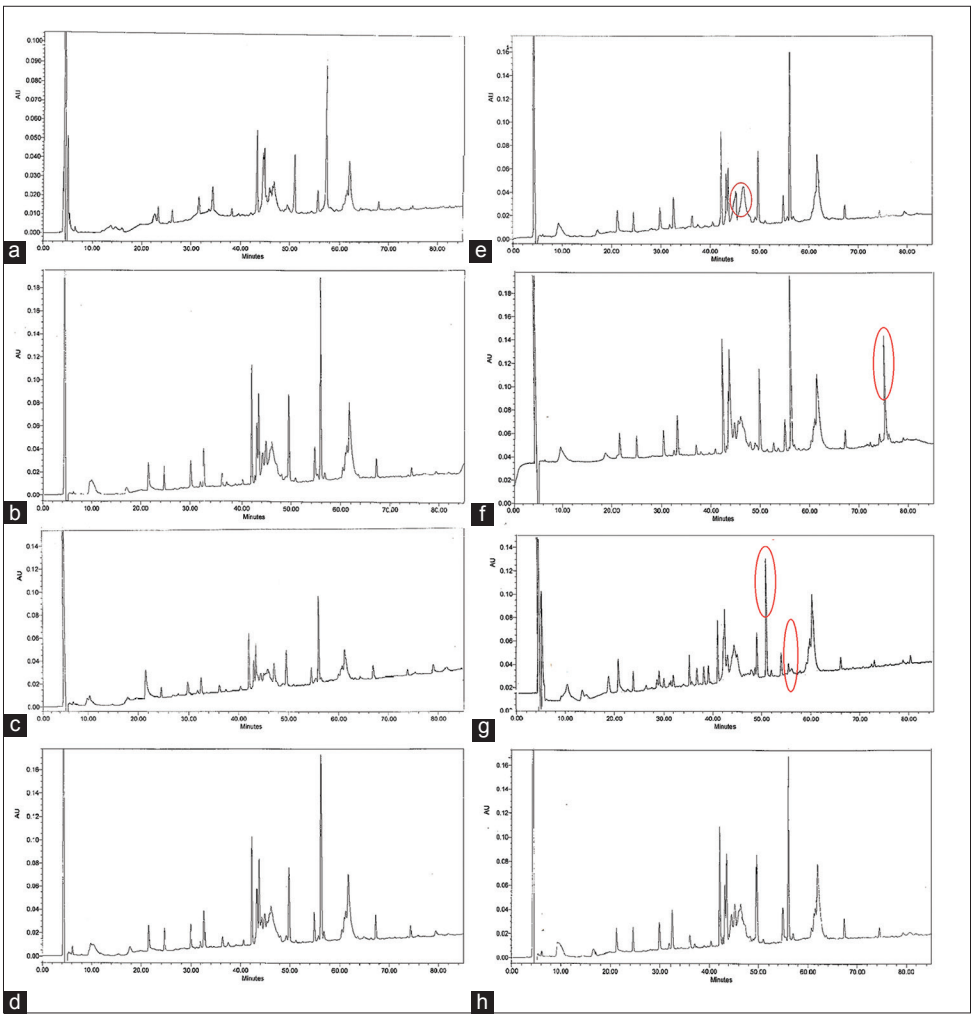

Figure 2: Peptide mapping profiles of different samples. (a) Under stress purified EPO; (b) Purified EPO exposed to room temperature stress; (c) Purified EPO exposed to pH4; (d) Purified EPO exposed to pH5; (e) Purified EPO exposed to acid hydrolysis; (f and g) Two rejected production batches; (h) Standard EPO

temperature) and partial deglycosylation on purified EPO were also studied. Peptide mapping analysis was performed for all samples and two samples of rejected production batches and compared with standard erythropoietin (shown in Figure 2).

Figure 3 shows the effect of various stresses imposed on molecular weight of purified EPO. As it can be seen from the SDS-PAGE electrophorogram [Figures 1 and 3], the bands are not sharp, and they seem like smears since EPO is composed of isoforms with different lengths of glycosylation. Lane E reveals the partial hydrolysis of glycosylated EPO where there is a reduction in the molecular weight of EPO as compared with intact EPO (lane A).

DISCUSSION

Trypsin cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine with an inability to cleave mentioned residues adjacent to proline,[52] thus the following cleaved peptide will be separated and eluted in order of increasing hydrophobicity by reverse phase chromatography.[53]

According to BP, acceptance limit for this test is full compliance of the chromatogram profile with the peptide mapping result of standard EPO. Comparing partially acid hydrolysis of purified EPO [Figure 2e] with standard [Figure 2h] depicts changes in peptide mapping of hydrolyzed EPO at retention time of 45 min. Differences in acid hydrolysis chromatogram is related to cleavage of glycosylated branches, which is similar to the deviations detected in an isoform with the lack of *N*-glycosylation (N24 and N38) and *O*-glycosylated (S126) residues in a similar work.[24]

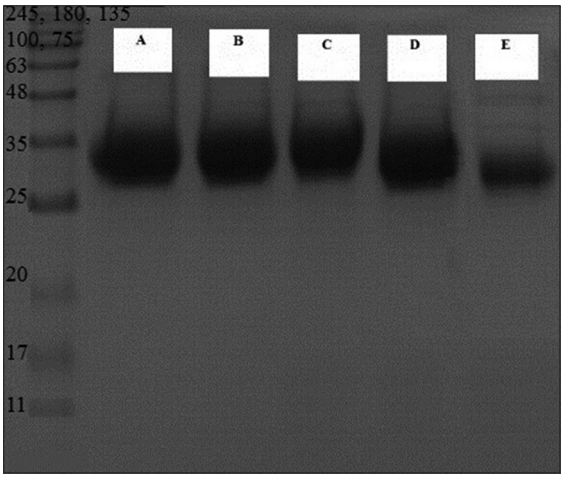

Figure 3: SDS-PAGE of intact and under extra stresses EPO. Intact EPO (lane A), purified EPO exposed to room temperature stress (lane B), pH 5 stress (lane C), pH 4 stress (lane D), and acid hydrolysis (lane E)

As it can be seen from Figure 2e at the retention time of 45 min, there is a peak that is absent in the other chromatograms. In the case of under stress purified EPO and extra exposure of purified EPO to acetate buffer pH 4, pH 5, and room temperature, no changes in the retention time or shape of the peaks could be observed [Figure 2a-d]. Figure 2f and g represent the peptide mapping profiles of two rejected batches, which had some variations during their cell culture steps. In Figure 2f, an extra peak was detected in the retention time of 77th min and in Figure 2g, many extra small peaks in retention time of 10–40 min, a main extra peak at 51st min and omitted peak at 56th min were detected.

In conclusion, no differences in peptide mapping among erythropoietin exposed to pH and temperature stresses and standard form were observed. This indicates that purification process of erythropoietin does not have any influences on its peptide mapping profile changes, and the stresses of purification process could not be the reason for rejection of products by peptide mapping. There might be some deviations in production steps other than purification process causing alteration in protein structure.

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