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Analysis of the Structure and Stability of Erythropoietin by pH and Temperature Changes using Various LC/MS

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The purpose of stability testing is to provide evidence about how the quality of a drug varies with time under the influence of a variety of environmental factors. In this study, erythropoietin (EPO) was analyzed under different pH (pH 3 and pH 9) and temperature (25 ºC and 40 ºC) conditions according to current Good Manufacturing Practice (cGMP) and International Conference on Harmonisation (ICH) guidelines. The molecular weight difference between intact EPO and deglycosylated EPO was determined by SDS-PAGE, and aggregated forms of EPO under thermal stress and high-pH conditions were investigated by size exclusion chromatography. High pH and high temperature induced increases in dimer and high molecular weight aggregate forms of EPO. UPLC-ESI-TOF-MS was applied to analyze the changed modification sites on EPO. Further, normal-phase high-performance liquid chromatography was performed to identify proposed glycan structures and high pH anion exchange chromatography was carried out to investigate any change in carbohydrate composition. The results demonstrated that there were no changes in modification sites or the glycan structure under severe conditions; however, the number of dimers and aggregates increased at 40 ºC and pH 9, respectively.

Key Words : Erythropoietin, SEC-HPLC, UPLC-ESI-Q TOF-MS/MS, HPAEC-PAD, NP-HPLC

Introduction

Erythropoietin (EPO), which is a well-characterized glycoprotein hormone, is used to illustrate the applicability of analytical methods for the characterization of proteins. It is the major physiological regulator of red blood cell formation, is produced primarily by the kidneys, and is excreted in the urine. The production of EPO is stimulated under hypoxia and it exerts its biological effect by binding to specific receptors on erythroid progenitor cells in the bone marrow. Human EPO has an apparent molecular weight of 30,000 Da, consists of 165 amino acids, and contains two disulfide linkages. The carbohydrate moiety of EPO makes up comprises approximately 40% of its molecular weight and consists of 4 glycosylation sites.[1] In addition, EPO is a glycoprotein that contains 3 *N*-glycans and a single *O*-glycan.[2] Glycosylation is a major source of structural complexity, heterogeneity, and variability of biopharmaceuticals.

The carbohydrate proteins of biopharmaceutical glycoproteins are considered to be critical attributes of the biological functions of these proteins since they are known to play an important role in protecting their peptide cores from modification.[3] Higher temperature and pH conditions EPO to initially dimerize, followed by high molecular aggregates. Aggregates of therapeutic proteins may compromise their safety and efficacy. The primary concern is that aggregates in therapeutic proteins may induce immune responses, which can have consequences ranging from reduction of product efficacy to patient fatality.[4] Therefore, in characterization studies, analyses of glycan structures are conducted by using various analytical techniques that include state-of-the-art mass technology to elucidate the detailed structures of carbohydrate side chains and their relationship with the biological potency of biopharmaceutical glycoproteins. Several methods are applied to characterize EPO by using high pH anion exchange chromatography (HPAEC-PAD) and NP-HPLC with mass spectrometry.[5] These include the structural characterization of glycoproteins, modifications of glycoproteins, composition analysis of released neutral glycans, and structural analysis of glycans.[6]

The higher complexities of peptides and proteins compared to organic low molecular weight drugs and the different ways that can be used to produce biotechnological products necessitates special requirements concerning quality assurance and analytical testing.[7] The activities of peptides and proteins depend not only on their amino acid sequences (primary structure) but also on their structures, which are determined by S-S linkages as well as hydrophobic and ionic interactions. A change in structure and conformation can significantly affect the potency of a substance or even lead to a complete loss of activity. Therefore, structural analysis is not only important during the development and evaluation of a new biotechnological drug but is also a decisive aspect in the quality control of therapeutically applied proteins. In most cases, a combination of different physicochemical, immunochemical, and biological techniques is necessary to carry out the comprehensive characterization and quality control of pharmaceutical peptide and protein drug substances. Many natural and recombinant proteins undergo posttranslational modifications (*i.e.*, cleavage, glycosylation, or phosphorylation). These post-translational modifications are dependent on specific protein structure as well as on the

host cell and significantly influence the stability, pharmacokinetics, and in vivo activity of a protein.[8]

In this study, physicochemical investigations were conducted by using LC-MS/MS methodologies to characterize the structural identity, integrity, and stability of EPO under thermal stress, and eventually to contribute to the establishment of specifications for its optimum storage conditions.[9]

Experimental

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. EPO was obtained from LG Life Science (Korea). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on Bis-Tris precast gels (15% T, 1.5 mm, 10 wells, MOPS running buffer). Total EPO *N*-glycans were released by PNGase F (New England Biolabs, Hitchin, UK) from EPO total protein that was heated at 95°C for 3 min and then added to *N*-octylglucoside. Electrophoresis was performed at a constant voltage of 200 V for 55 min.[10] SDS-PAGE gels were fixed for 60 min in a solution that contained 7.5% acetic acid and 10% methanol. Subsequently, the gels were stained with Coomassie R-250 overnight and then destained for several hours in a solution consisting of 10% acetic acid and 20% methanol. The staining solution was prepared in 100 mL of a solvent containing 10% acetic acid and 45% methanol. The solution was freshly prepared and filtered (595 1/2 fold filters; Whatman; Dassel, Germany) before use.[11]

Size Exclusion Chromatography. Analyses of EPO drug aggregates were conducted by using a size-exclusion chromatography (SEC-HPLC) method for which a Waters Alliance 2690 HPLC system was connected to a TOSOH TSKgel G3000 SWXL column (7.8 mm × 30 cm, 250 Å pore size, 5 μm particle size) and a Waters spectrophotometer detector. Chromatographic control, data acquisition, and analysis were performed by using Empower software *via* a Waters LACE data acquisition box. The samples were stored refrigerated in an auto-sampler, which was part of the Waters Alliance 2690 Alliance system. The spectrophotometer UV detector was operated at a wavelength of 214 nm. The mobile phase (1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, and 0.4 M sodium chloride at pH 7.4) was filtered using a 0.45-μm filter (Millipore) and degassed with an online degasser. The flow rate was 0.5 mL/min and the column was maintained at ambient temperature. The method run time was a minimum 60 min. The injection volumes for the test samples and standards varied between 50 μL and 100 μL, depending on the sample concentration. The column was equilibrated with a minimum of 5 injections (50 μL) of sample diluent buffer until a stable baseline was obtained.[12]

Ultra-performance Liquid Chromatography-electrospray Ionization-quadrupole Time-of-flight Mass Spectrometry/mass Spectrometry. To examine changes in EPO modification sites, the EPO drug was desalted (Microcon YM-10) and reconstituted at a concentration of 1.5 μg/μL in 28 μL of water, and then mixed with 1 μL of Tris acetic buffer (pH 8.5, 1 M), 1 μL of protease trypsin (concentration 1 μg/μL, Merck, Darmstadt, Germany), and 42 μL of water. The mixture was incubated for 18 h at 37 °C and the digestion was stopped by heating the mixture at 100 °C (boiling water bath) for 1 min.[13]

Lyophilized peptides were reconstituted in 20 μL of 0.1% formic acid. Each sample was analyzed by carrying out independent experimental runs *via* LC/MS/MS using an ACQUITY ultra-pressure liquid chromatography (UPLC) and Synapt Q-Time-of-Flight (TOF) mass spectrometer that was equipped with a Lockspray ion source (Waters, Manchester, UK). A Mass PREP Digestion Standard (Protein Expression Mixture 1 & 2) (Waters, Massachusetts, USA) was run before and after the samples to monitor sensitivity and quantity. Five microliters of each sample was injected online onto a Waters Acquity UPLC BEH C18 column (2.1 × 150 mm length with 1.7 μm particle size).[14]

Peptides were separated by in-line gradient elution, at 300 μL/min using a linear gradient from 3-45% B over 15 min (A; 0.1% formic acid in water, B; 0.1% formic acid in acetonitrile), followed by 10 min rinses at 90% B. The column was re-equilibrated with 3% B for 5 min prior to the next run. All of the column temperatures were maintained at 35 °C. The mass accuracy was maintained during the run by using a lockspray of the peptide [glu1]-fibrinopeptide B that was delivered through the auxiliary pump of a NanoACQUITY at 400 fmol/μL and 5 μL/min. Peptides were analyzed in positive ion mode and the TOF analyzer was operated in V-mode with a typical resolving power of 10,000 fwhm. Prior to the analyses, the TOF analyzer was calibrated by using [glu1]-fibrinopeptide B fragments that were obtained using a collision energy of 30 eV and over the mass range 50-1990 *m/z*. The Q-TOF was operated in the LC/MS/MS acquisition mode. For each injection, the mass spectrometer acquired data from 0 to 35 min. Water BiopharmaLynx version 1.2 was used to process each raw data file. Each processed file was then searched against an EPO protein sequence database. Trypsin was selected as the proteolytic enzyme and a missed cleavage allowed. Glycosylation, deamidation, and oxidation were selected as fixed modifications after all of the EPO samples had been.[15]

HPAEC-PAD.

Preparation of Standard Mixtures: Stock standard solutions (100 mM) were prepared by dissolving each standard monosaccharide in a mixture of water. Working standard solutions were further obtained by appropriate dilution of the stock standard solution with deionized water. The sample solutions were filtered through a 0.22-μm syringe and degassed using an ultrasonic bath for 2 min prior to use. All of the solutions that were prepared were stored in the dark at 4 °C until they were used.[16]

Oligosaccharides were subjected to acid hydrolysis in the presence of 2 M HCl in sealed glass tubes at 100 °C for 2 h. After drying in a vacuum, the resulting monosaccharides were dissolved in water and then injected *via* an Autosampler System AS50 (Dionex). Reference monosaccharide mixtures (L-fucose, D-glucosamine and D-galactosamine,

and D-mannose, and D-galactose) were injected at different concentrations (10-100 pmol each) and the amounts of monosaccharides in the test samples were calculated from the specific HPAEC-PAD responses that were obtained for the reference standards.

Hydrolysis of *N*-/*O*-glycan. For neutral sugar analysis, 600 pmol of *N*-/*O*-glycans were dissolved in 400 μL of 2 M TFA and incubated at 100 °C for 4 h. The hydrolyzed glycans were dried using a Speed-Vac, dissolved in 100 μL of distilled water, and filtered with an Amicon Micro-EZ filter tube (Millipore, Bedford, MA), after which they were ready to be used in the following experiments.

HPAEC-PAD Equipment and Conditions. Analysis of the monosaccharides was carried out using a Dionex Bio-LC system that was equipped with a GP50 gradient pump unit, an ED50 electrochemical detector, an ASI-100 autosampler, and a LC30 chromatography enclosure that were controlled by using a Dionex CHROMELEON workstation. The analytical column was a CarboPac PA-1column (4.0 × 250 mm; Dionex, USA). Elution was carried out at 1.0 mL/min at room temperature. Mobile phase A consisted in 200 mM NaOH and mobile phase B was deionized water. The injection volume was 10 μL.

Normal-phase High-performance Liquid Chromatography. To examine changes that occurred in the *N*-glycan structures under stress conditions, the total erythropoietin *N*-glycans of EPO were cleaved and released by PNGase F (New England Biolabs, Hitchin, UK) from the erythropoietin total proteins and analyzed by normal-phase (NP)-HPLC analysis after fluorescent labeling of *N*-glycans with 2-aminobenzamide (2-AB). One hundred micrograms of each of the lyophilized total proteins was dissolved in distilled water (28 μL) and denatured at 100 °C for 10 min with 10 μg of denaturing buffer (New England Biolabs, Hitchin, UK) according to the manufacturer's instructions. After cooling, 42 μL of ddH_2O, 10 μL enzyme buffer (as supplied by the manufacturer), 10 μL of 10% NP40, and PNGase F (1000 U/μL) were added and the mixture was incubated at 37 °C for 18 h. The proteins were precipitated by adding 400 μL of cold acetone and removed by centrifugation at 15,000 × *g* at 4 °C for 5 min. The oligosaccharides that were obtained were mixed with 100 μL of cold 60% methanol, sonicated gently, and allowed to stand at −20 °C for 1 h. The supernatant containing the oligosaccharide was then applied to a C18 cartridge (Sigma Aldrich) to remove any soluble peptides and then dried in a Speed-Vac.[17]

The dried *N*-glycans that were obtained were labeled with 2-AB by reductive amination according to the method of Bigge et al. using a 2-AB labeling kit (Sigma Aldrich). The 2-AB-labeled *N*-glycans were dissolved in distilled water and analyzed on a TSK-GEL Amide 80 column (4.6 × 250 mm; TOSOH Bioscience LLC, Japan).[18]

Normal-phase high-performance liquid chromatography (NP-HPLC) was carried out using a P680 HPLC pump system that was equipped with a RF 2000 fluorescence detector (DIONEX. USA). The oligosaccharides were eluted in the following gradient mode with solvent A (50 mM ammonium formate at pH 4.4) and solvent B (acetonitrile): 20% A at 0.4 mL/min, then a linear gradient of 20-58% A for 152 min, and finally 58-100% A over the next 3 min. The flow rate was then increased to 1 mL/min over the next 2 min and the column was washed in 100% A for 5 min before being re-equilibrated with 20% A for the injection of the next sample. The relative fluorescence was measured at Em 420 nm and Ex 330 nm. The relative amounts of the total major glycans that were contained in each peak (Pk1-6) were calculated from the peak areas (mean of 2 independent analyses). The sum of the areas of peaks 1-6 was set to 100%. The GU values were determined by reading the elution position of each glycan against a standard curve that was derived from the elution positions of a standard mixture of glucose oligomers (dextran ladder: tetramer to 12-mer glucose residues). Calculation of the GU value was referenced from the report (GlycoBase) and the proposed structures of the *N*-glycans in each peak were estimated.

Results and Discussion

Identification of Deglycosylated and Intact EPO by SDS-PAGE. The proteins were treated with PNGase-F in order to obtain deglycosylated forms and then analyzed by SDS-PAGE. As can be seen in Figure 1, the EPO protein that was treated with PNGase-F was deglycosylated, thereby resulting in a decrease in its molecular weight of approximately to 18 kDa (Lane 3) compared to intact EPO at approximately 30 kDa (Lane 2).

Identification of Aggregation Stability by Size-exclusion Chromatography. Size-exclusion chromatography (SEC-HPLC) is used to control for the absence of fragments and aggregates. This technique was applied to characterize erythropoietin dimerization that occurs at high storage temperatures and under various formulation conditions.

The EPO drug is a relatively stable glycoprotein hormone that remains in monomeric form when it is stored under

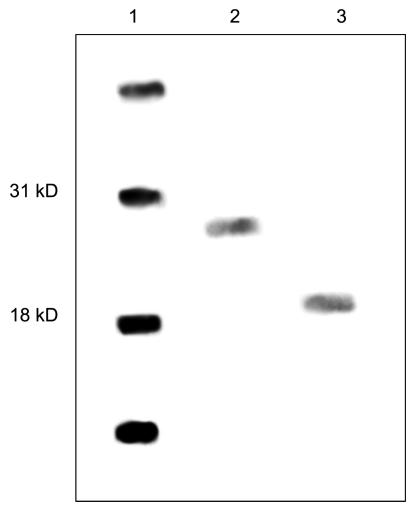

Figure 1. SDS-PAGE results of native erythropoietin (EPO) and deglycosylated EPO. Lane 1: Molecular weight marker; lane 2: Intact EPO; and lane 3: EPO treated with PNGase-F.

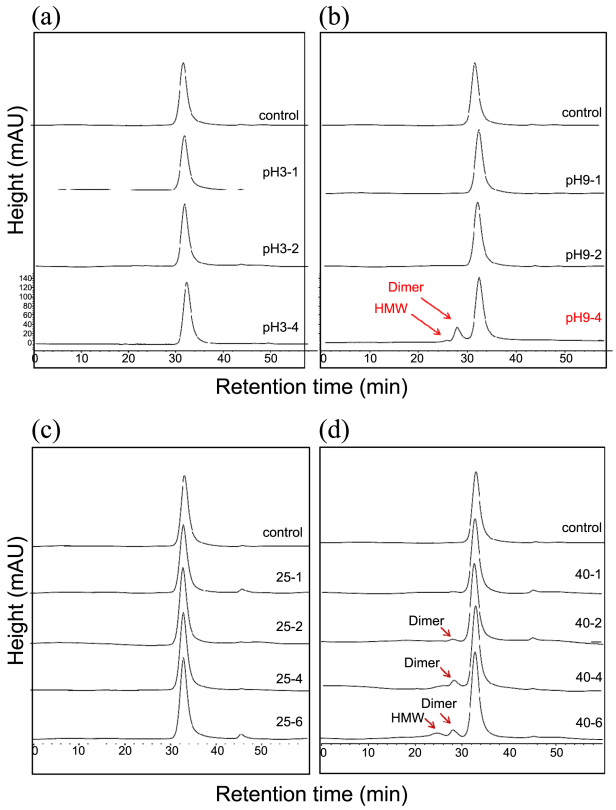

Figure 2. Size-exclusion chromatography chromatograms of erythropoietin samples that were subjected to pH [pH 3(a) of pH 9(b)] and temperature [25 °C (c) or 40 °C (d)] stress tests for 1, 2, 4, and 6 wks.

refrigeration. Its monomeric integrity is routinely monitored by SEC-HPLC. When the EPO drug was stored at pH 7 (control), no aggregates were detected by SEC-HPLC. At pH 3, which is a severe condition (5 °C ± 2 °C), it was monitored in terms of maintaining its monomeric integrity (Figure 2(a)). The high pH condition (5 °C ± 2 °C) induced EPO to initially dimerize, which was followed by the formation of higher molecular weight aggregates. In particular, not only was the degree of aggregation increased at pH 9 for 4 wks, but the degree of dimerization and formation of high molecular weight aggregates also increased (Figure 2(b)).

The EPO drug was also stored either at −20 °C or −70 °C but no aggregates were detected by SEC-HPLC, even after 6 wks. In order to investigate the thermal stability of EPO protein, stress tests were conducted at accelerated temperature conditions (25 °C ± 2 °C/60% RH ± 5% RH) and extreme temperature conditions (40 °C ± 2 °C/75% RH ± 5% RH). The test results at 25 °C showed that there was no evidence of aggregation of the EPO protein, even after 6 wks (Figure 2(c)). In contrast, the stress of 40 °C induced the formation of dimerized higher molecular weight aggregates (Figure 2(d)). As can be seen in Figure 2(c) and Figure 2(d), in particular, dimers were generated during the first 2 wks but increased at 40 °C at 4 to 6 wks.

In general, the unglycosylated proteins are prone to aggregation because of its high content of nonpolar amino acids. Heat- or pH-induced aggregation of EPO (as described above) may therefore have been due to exposure of hydrophobic regions of the protein (Table 1, and Table 2(a) and 2(b)).

Table 2. Size-exclusion chromatography chromatograms of erythropoietin under accelerated conditions of 25 °C (a) or 40 °C (b) for 1, 2, 4, and 6 wks, respectively

(a)

Condition	Peak	Retention time (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area
1 wk	1	32.791	1.6914	1.52351e4	121.79	100.00
2 wks	1	28.12	1.08	242.32	2.67	1.49
	2	32.80	1.72	1.60213e4	129.02	98.51
4 wks	1	24.71	1.19	185.75	1.82	1.18
	2	28.12	1.09	787.56	8.49	5.02
	3	32.79	1.74	1.46848e4	118.43	93.78
6 wks	1	25.85	1.30	370.28	3.34	1.77
	2	28.00	1.30	1474.64	13.29	7.07
	3	32.70	1.76	1.9011e4	148.45	91.15

(b)

Condition	Peak	Retention time (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area
Control	1	32.81	1.71	2.16270e4	168.61	100.00
1 wk	1	32.75	1.73	1.82720e4	145.54	100.00
2 wks	1	32.77	1.79	1.8007e4	142.78	100.00
4 wks	1	32.79	1.82	1.93599e4	152.24	100.00
6 wks	1	32.82	1.63	1.77011e4	139.07	100.00

Table 1. Size-exclusion chromatography chromatograms of erythropoietin under accelerated conditions of pH 3 or pH 9 for 1, 2, and 4 wks, respectively

Condition	Aggregation	Retention time (min)	Width (min)	Height (mAU)	Area
pH 3-1	Monomer	32.79	1.77	153.77	100.00
pH 3-2	Monomer	32.80	1.76	160.62	100.00
pH 3-4	Monomer	33.32	1.75	153.31	100.00
pH 9-1	Monomer	32.81	1.78	156.70	100.00
pH 9-2	Monomer	32.79	1.77	162.59	100.00
pH 9-4	High Molecule Weight	26.22	0.77	1.52	0.72
	Dimer	28.43	1.21	19.62	14.55
	Monomer	33.11	1.74	92.79	84.72

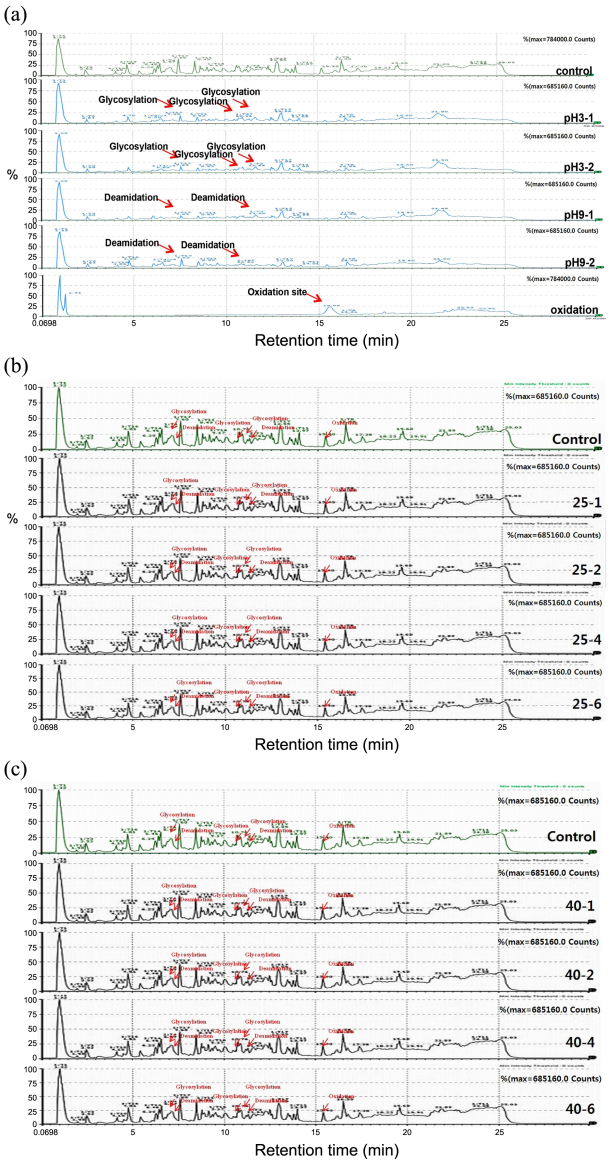

Figure 3. Ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry/mass spectrometry quantification of erythropoietin samples in pH stress tests (a) and temperature [25 °C (b), 40 °C (c)] stress tests for 1, 2, 4, and 6 wks. Five microliters of each sample was injected online onto a Waters Acquity UPLC BEH C18 column (2.1 × 150 mm length with 1.7 μm particle size). Peptides were separated by in-line gradient elution at 300 L/min by using a linear gradient from 3% to 45% B over 15 min (A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile) followed by 10 min rinses at 90% B. The column was re-equilibrated with 3% B for 5 min prior to the next run. All column temperatures were maintained at 35 °C.

Characterization of EPO Modification sites Under Various pH and Temperature Conditions. Direct anlaysis of glycopeptides by mass spectrometry (MS) represents a rapid and sensitive method to obtaion site-specific characterizations of glycoproteins. It can provide information on the modification sites of glycoproteins and on peptide sequences within a single experiment.

As can be seen in Figure 3(a)-3(c), the characterization was conducted by using a novel ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry/mass spectrometry system to thoroughly identify any changed modification sites (*i.e.*, through glycosylation, deamidation, or oxidation).

The results demonstrate all EPO drugs under temperature and pH stresses that not modified all modification site (glycosylation, deamidation, and oxidation) and represented same retention time (Table 3 and Figure 4). A deamidation site was identified for high pH stress and an oxidation site was identified for oxidation stress.

Analysis of Proposed *N*-Glycan Structure Under Various Temperature Conditions by NP-HPLC. Normal-phase HPLC using amide-based columns is a robust and reproducible method for carrying out the high-resolution separation of *N*-linked glycans that are released from glycoproteins. The released glycans labeled with 2-aminobenzamide, which enables their detection at the femtomole level. NP-HPLC can separate structures that have the same composition on

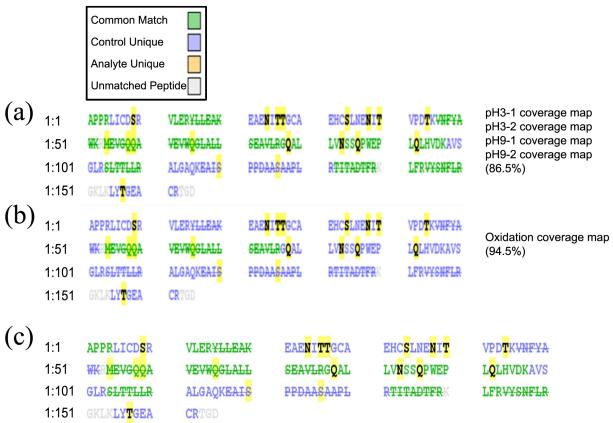

Figure 4. Coverage map comparison of ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry/mass spectrometry analysis of testing samples. (a) pH coverage map, (b) oxidation coverage map, and (c) temperature coverage map.

Table 3. Peak match data of modification site under temperature, pH, and oxidation stresses

Peptide	Fragment number	Start	End	Modification	Calculated peptide mass (Da)	b/y possible	Control retention time (min)	Control *m/z*
LICDSR	1:T2-1:T5-1:T20	5	10	Glycosylation	6604.72	70	10.8	1652.19
EAISPPD	1:T13	117	131	Glycosylation	1667.83	28	6.9	834.93
LICDSR	1:T2-1:T5-1:T20	5	10	Glycosylation	6313.68	70	10.6	1579.42
GQALLVN	1:T9	77	97	Deamidation	4129.82	40	10.9	1033.49
MEVGQQ	1:T8	54	76	Deamidation	2527.29	44	7.1	843.43
MEVGQQ	1:T8	54	76	Oxidation	2541.32	44	15.9	848.12

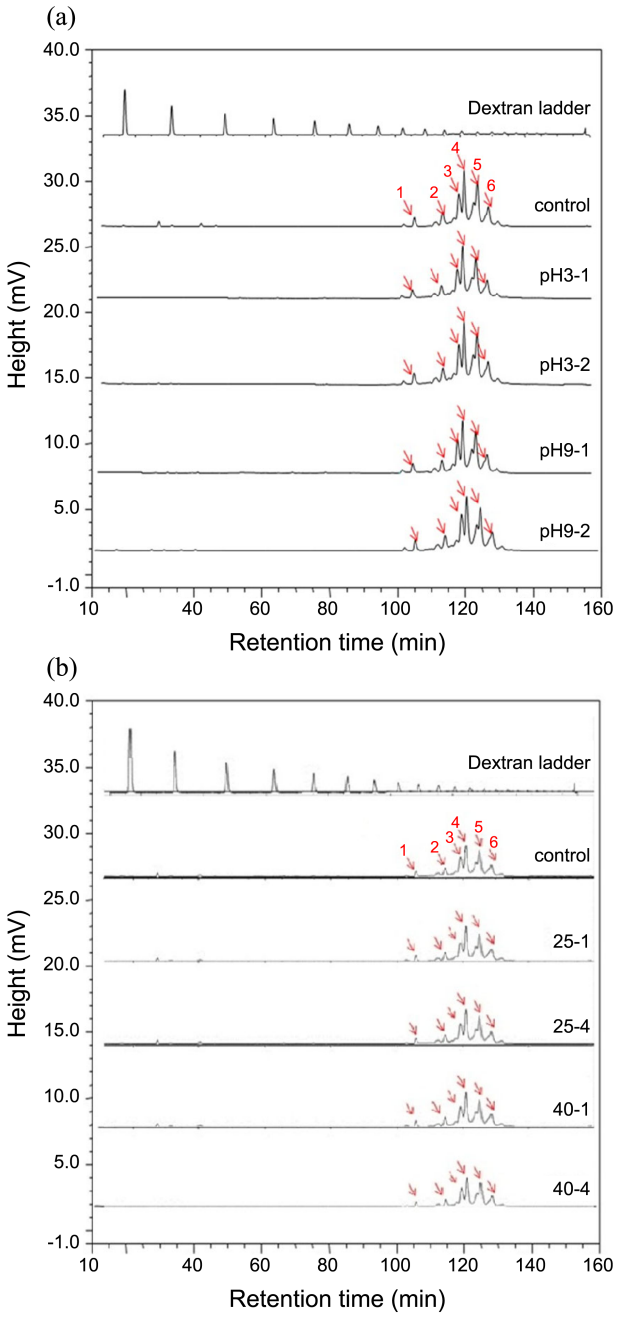

Figure 5. Normal-phase HPLC chromatogram of desialylated N-linked glycans obtained under different pH (a) and temperatures (b) conditions. A TSK-GEL Amide 80 column (4.6 × 250 mm TOSOH Bioscience LLC, Japan) was used. NP-HPLC was carried out using a P680 HPLC pump system that was equipped with a RF 2000 fluorescence detector (DIONEX. USA). The oligosaccharides were eluted in the following gradient mode with solvent A (50 mM ammonium formate at pH 4.4) and solvent B (acetonitrile): 20% A at 0.4 mL/min followed by a linear gradient of 20-58% for 152 min and finally 58-100% over the next 3 min. The flow rate was then increased to 1 mL/min over the next 2 min and the column was washed in 100% A for 5 min before being re-equilibrated with 20% A for injection of the next sample. The relative fluorescence was measured at Em 420 nm and Ex 330 nm.

Table 4. All of the stressed samples showed the same proposed glycan structure compared to the control under different temperature conditions (a) and pH conditions (b)

(a)

Peak number	Retention time (min)	GU value	Proposed glycan structure
1	105.69	7.5	
2	114.55	8.39	
3	119.36	9.26	
4	120.96	9.39	
5	124.93	9.51	
6	128.44	9.86	

(b)

Peak number	Retention time (min)	GU value	Proposed glycan structure
1	105.69	8.03	
2	114.55	8.46	
3	119.36	9.54	
4	120.96	9.88	
5	124.93	10.04	
6	128.44	11.8	

the basis of sequence and linkage type (MS analysis can be used to distinguish differences when employed in combination with chromatographic separation and/or fragmentation analysis).

To analyze the *N*-glycan structures of EPO under various temperature and pH stresses, NP-HPLC was performed.

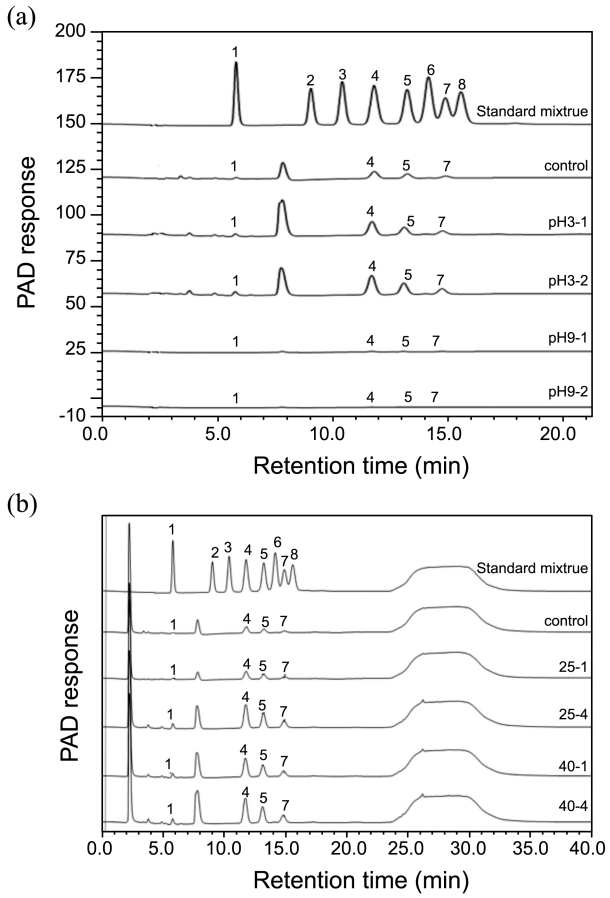

Figure 6. High pH anion exchange chromatography with pulsed amperometric detection chromatogram of carbohydrate composition of pH-stressed EPO (a) and temperature-stressed EPO (b). Standard mixture: 1. Fuc, 2. Ara, 3. Rha, 4. GlcNAc, 5. Gal, 6. Glu, 7. Man, and 8. Xyl. The analytical column that was used was a CarboPac PA-1column (4.0 × 250 mm, Dionex, USA). Elution was carried out at 1.0 mL/min at room temperature.

Glycan structures were determined based on glucose unit (GU) values. The elution times that were obtained for the different glycan peaks were converted in to GU by making comparisons with the elution times of fractions that were obtained from a dextran ladder. This allowed for the structural assignment of these peaks by making comparisons with the GU values that were obtained from the standards. Through these steps, six Peaks were detected, and the chromatograms that were obtained for each sample resulted in the same profiles with characteristic retention times or GU values (Figure 5(a) and 5(b)). Using the GU values, the *N*-glycan structures that we proposed were confirmed by using Glycobase 2.0.

The temperature-stressed samples showed that GU 7.05 has an F(6)A2GBG1GA1 structure, GU 8.39 has an F(6)A2G2S(3)2 structure, GU 9.26 has an F(6)A2BG2S(6)2 structure, GU 9.39 has an F(6)A2BG2S(6)2 structure, GU 9.51 has an F(6)A2G1GA1 structure, and GU 9.86 has an F(6)A4BG3 structure.

The pH-stressed samples showed that GU 8.03 had F(6)A3[6]BG(4)1, GU 8.46 had A2[3]G(4)25(6)2, GU 9.54 had F(6)A2F(3)2G(4)2F(2)1, GU 9.88 had A3F(3)2G(4)3, GU 10.04 had F(6)A4G4 and GU 11.8 had M9Glc3 structures, respectively (Table 4(a) and 4(b)). All of the samples contained hexose (galactose, fucose, and mannose) and *N*-acetylglucosamine.

Table 5. Changed carbohydrate compositions of erythropoietin under stress conditions of temperature (a) and pH (b) by using HPAEC-PAD

(a)

	Control (nmol)	25–1 w (nmol)	25–4 w (nmol)	40–1 w (nmol)	40–4 w (nmol)
1. Fuc	0.25	0.33	1.10	0.82	1.13
4. GlcNAc	1.82	2.25	7.30	5.60	7.51
5. Gal	1.10	1.51	4.84	3.66	4.92
7. Man	0.68	0.86	3.00	2.30	3.10

(b)

	Control (nmol)	pH 3–1 (nmol)	pH 3–2 (nmol)	pH 9–1 (nmol)	pH 9–2 (nmol)
1. Fuc	0.25	0.47	0.68	0.01	0.01
4. GlcNAc	1.82	3.21	4.60	0.05	0.08
5. Gal	1.09	1.98	2.90	0.04	0.07
7. Man	0.67	1.21	1.74	0.03	0.04

Composition Analysis of N-linked Glycans in EPO by using HPAEC-PAD. The accurate analysis of the glycosylation patterns of glycoproteins is essential when investigating potential disease-specific changes and their subsequent evaluation as biomarkers of disease diagnosis, prognosis, and progression. HPAEC is capable of providing information on both the monosaccharide and the oligosaccharide composition of N-linked oligosaccharides. A major advantage of this technique is that it does not require prior derivatization or clean-up of carbohydrate samples in order to allow quantification. One of the first columns that were developed for this, CarboPac PA1, was a multipurpose column that could be used for the analysis of monosaccharides, disaccharides, and specific oligosaccharides, as shown Figure 6. Before carrying out a ratio analysis, standard monosaccharide mixtures were prepared using neutral sugars and amino sugars under various temperature and pH conditions. Compared to the mixture, fucose, *N*-acetylglucosamine, galactose, and mannose were identified. In particular, none of the samples showed changed sugar contents compared to the control (Table 5(a) and 5(b)). These results demonstrate that there was no effect on the EPO sugar chains due to the temperature and pH stresses.

Conclusion

EPO has a heterogeneous structure because its carbohydrates vary in amount and size. It is of great importance to establish processing and storage conditions for which its conformational integrity is well maintained. Thorough investigations were conducted by using various physicochemical

methods, including LC-MS/MS, in order to characterize the structural identity, purity, integrity, and stability of EPO protein under temperature and pH stresses. Direct anlaysis of glycopeptides by MS represents a rapid and sensitive method to obtain site-specific characterizations of glycoproteins. It can provide information on the modification sites of glycoproteins and on peptide sequences within a single experiment. Detailed analytical procedures were referenced in monographs for EPO drug by the Korean Food and Drug Administration and the European Pharmacopoeia.

In this study, intact EPO and deglycosylated EPO were stored under various temperature conditions (25 °C and 40 °C) and pH conditions (pH 3 and pH 9) over 6 wks. Our test results for SEC-HPLC showed that dimer and HMW aggregate forms of EPO increased under temperature and pH conditions of 40 °C and pH 9, respectively. It is conceivable that unglycosylated EPO is prone to aggregation at high temperature and pH induces exposure of hydrophobic regions of the protein. To analyze any changes in EPO modification sites, stress tests were performed. The test results revealed that there were no changes in the modification sites, which suggests that the EPO disulfide bond bridges (Cys-Cys) were maintained under the varying temperature and pH conditions. *N*-glycans were analyzed using both NP-HPLC and HPAEC-PAD. The test results demonstrated that there was no significant change either in the proposed carbohydrate structure of EPO or in the quantity of individual monosaccharide in EPO.

On the basis of the results of this study, stress testing of the drug substance can help identify probable degradation products. This can in turn help to establish storage conditions and the intrinsic stability of molecule and validate the stability indicating power of the analytical procedures used.

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