Erythropoietin Biosimilar Characterization

Demonstration of analytical, pharmacokinetic and therapeutic equivalency of a biosimilar with its reference product is essential for its regulatory approval. In particular, analytical characterization studies demonstrating similarity are crucial part of a marketing authorization application. Though 'complete' analytical characterization is an interminable activity, rigorous and orthogonal analytical techniques can provide a comprehensive understanding of biosimilar with respect to innovator's product. As a biopharma solution provider, Shantani focuses on providing an extensive understanding of the target product profile from an analytical stand point of view. We utilize cutting-edge orthogonal approaches for product characterization, and without compromising the quality and integrity of the data, we provide results in a format that is comprehensible both to the manufacturer and the regulators alike. The present study that characterize some physico-and immuno-chemical properties of Erythropoietin Biosimilar showcase our analytical expertise.

Erythropoietin is a hormone secreted by interstitial kidney fibroblasts in response to cellular hypoxia, to stimulate the production of red blood cells in the bone marrow. Exogenous erythropoietin or recombinant human erythropoietin (rhEPO) and its analogues, collectively called erythropoiesis-stimulating agents (ESA) has been used for the treatment of anemia, in patients with chronic kidney disease and cancer chemotherapy for more than two decades.

Saba Naaz Siddique
Director-Sales
saba@shantani.com

Purva Satpute
Sr. Research Associate
psatpute@shantani.com

Chaitanya Saxena
CEO
csaxena@shantani.com

Shantani Proteome Analytics Pvt., Ltd.
Pune

Structurally, Human EPO is an acidic glycoprotein with a molecular mass of approximately 30.4 kDa. Its 165 amino acid residues chain forms four antiparallel α-helices, two β-sheets and two intra-chain disulfide bridges (Cys^{7}-Cys^{161}, Cys^{29}-Cys^{33}). The carbohydrate portion (40% of the molecule) comprises three N-glycans (at Asn^{24}, Asn^{38}, and Asn^{83}) and one O-glycan (at Ser^{126}). The variety of glycosylation patterns give rise to alpha, beta, delta, and omega erythropoietin forms.

These glycosylation isoforms of EPO and its analogs can be distinguished by isoelectric focusing and immunoblotting. *(Reference: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3822280/)*

The global erythropoietin (EPO) drugs market size was valued at USD 7.4 billion in 2016 and is expected to witness CAGR of 11.5% during the forecast period (2014 - 2025). *(Reference - https://www.grandviewresearch.com/industry-analysis/erythropoietin-epo-drugs-market)*. Patent expiration of major biologics created opportunities for the entry of erythropoietin biosimilars in the market. Binocrit (Sandoz) was the first Epoetin alpha biosimilar approved in Europe in July 2007. Following this, several Erythropoietin biosimilars were launched by major companies such as Biocon, Ranbaxy, Emcure Pharmaceuticals. EPO biosimilars have been marketed in the European Union for over a decade. Retacrit, developed by Hospira, a Pfizer company, was the first epoetin zeta biosimilar approved in the United States in May 2018.

Several epoetin biosimilars are under different stages of development at various companies around the world. Approval of these biosimilars will primarily depend on establishing their similarity with the reference drug by performing extensive structural and functional characterization. Technology solution providers like 'Shantani' who supports the product characterization requirements of biopharma product developers provides a one-stop solution for fulfilling the program's regulatory requirements.

Shantani's consultation-characterization-collaboration business model spans across lab-to-regulators-to-market assuring best quality services to the collaborators. To briefly showcase our analytical capabilities, we are sharing details of a small study that was performed to confirm the identity, integrity and purity of the erythropoietin concentrated solution.

Immunoblotting

Methodology

Protein equivalent to 2.0 µg was mixed with 1X LDS sample buffer and 50 mM DTT. Samples were heated in a dry bath at 95 degree centigrade for 10 minutes. Samples were vortexed and briefly centrifuged. Protein equivalent to 1.0 µg was used for SDS-PAGE followed by western blot analysis. Proteins in SDS-PAGE gel were visualized by using CBB dye. For immunoblotting, both primary and secondary antibody were used at a dilution of 1:1000. Secondary antibody conjugated with HRP and associated peroxidase reaction leading to chemiluminescence was used for visualizing the protein bands.

Results, Interpretations and Discussions

In SDS-PAGE analysis a clear and single protein band was observed in the molecular weight range of 30 to 40 kDa. Typical molecular weight of the EPO is known to be in the same range. EPO specific antibody was also used to probe the de-stained protein bands. Antibody specific interaction and molecular weight of protein located in 30 to 40 kDa region confirmed the identity of protein to be Erythropoietin. A representative image of gel-code blue stained gel and immunoblotted protein band is shown in Figure 1.

Conclusion

Samples when probed with anti-Erythropoietin antibody using immuno-blotting method, provided positive confirmation of presence of Erythropoietin.

Peptide Mapping using LC-MS/MS

Methodology

Deglycosylated Erythropoietin Concentrated sample was acetone precipitated to prepare 100 µg equivalent protein sample. Sample equivalent to 30 µg of protein was denatured with 8M urea. Samples were further reduced by adding DTT and incubating at 90˚C for 15 minutes. Samples were cooled and alkylated using iodoacetamide. Protease trypsin was added in the ratio of 1:20 (Protein:Substrate) and proteolysis was carried out for 16 hours at 37˚C. Digestion was stopped by adding 100% trifluoroacetic acid (TFA). In a parallel analysis, protein samples were digested with Chymotrypsin in a calcium chloride buffer.

Mass-Spectrometry based Analysis: Protease digested peptides were concentrated close to dryness and were dissolved in 0.1% TFA. 5 µl of this sample was injected onto HPLC-Chip/MS system, a microfluidic chip-based technology that incorporates peptide enrichment and separation, and MS and MS/MS data of peptides were acquired in data dependent mode. MS data acquired were extracted using Morpheus software for matching the MS and MS/MS information of peptides. The information was compared

The different types of ESA's and their originator products are listed in Table 1:

Sr. No.	Exogenous ESA's	Originator Product	Manufacturer/License Holder(s)	Patent Expiry
1.	Epoetin alfa	Epogen/Eprex/ Procrit	**Epogen** (approved in 1989) was manufactured and sold by Amgen. It was licensed to Johnson & Johnson/Ortho Biotech to be sold outside US (excluding Japan) under the brand name **Procrit/Eprex**	Patent Expired both in the US and in Europe in 2013
2.	Epoetin beta	NeoRecormon/ Epogin	**Recormon®** (approved in Europe in 1990) was developed by and manufactured by Boehringer Mannheim. It was reintroduced as **NeoRecormon®** in Europe by Roche in 1997 and marketed as Epogin by Chugai in Japan.	Patent Expired in Europe in 2005
Second Generation EPO				
3	Darbepoetin alfa	Aranesp	Amgen's **Aranesp,** a modified hyperglycosylated epoetin was approved by USFDA in September 2001 and by EMA in August 2001	Patent expired in US in May 2024 and in Europe in July 2016
4.	Methoxy PEG epoetin-beta	Mircera	Developed and marketed by Roche, **Mircera** was approved by FDA and EMA in 2007	Will expire in US on June 2020
Third Generation EPO				
5.	Epoetin Theta	Eporatio/Biopin	**Epoetin theta** was developed by Merckle Biotec (Ulm, Germany) and was approved by EMEA on October 23, 2009. It was marketed as **Biopoin®** by CT Arzneimittel and as **Eporatio®** by Teva (Ratiopharm)	N.A.
6.	Epoetin delta	Dynepo	**Epoetin delta**, the only EPO synthesized in a human cell line using Gene Activation Technology (Transkaryotic Therapies, Inc.) was approved by EMA in 2002. Marketing authorization was held by Shire Pharmaceutical which was withdrawn in 2009 for commercial reasons.	N.A.

Table 1. *Different types of ESA's and their Original Product*

against the known amino acid sequence of Erythropoietin. Peptide Identification was performed with the following criteria: (a) Trypsin and chymotrypsin digested peptides with 4 missed cleavages allowed, (b) peptide tolerance < + 2.1 Da, (c) product tolerance < + 0.025 Da, (d) False Detection Rate (FDR) < 1%.

Results, Interpretations and Discussions

Probable tryptic peptides that can be obtained from the EPO are shown in the schematic (Figure 2). From the Trypsin digest, an 80% coverage of the known amino acid sequence of the EPO protein confirmed the identity of the protein sample to be Erythropoietin. Peptides that were not identified are either known to be glycosylated and hence their peptide mass didn't match, or these peptides were very hydrophilic and small and hence were not captured on C-18 resins. To confirm the same, EPO protein was first deglycosylated and later digested with Chymotrypsin. Probable chymotryptic peptides that can be obtained from the EPO are shown in the schematic (Figure 3). A coverage map of identified peptide is shown in Figure 4. When peptides identified from trypsin and chymotrypsin digest were combined it provided an 100% coverage of the known amino acid sequence of the EPO protein.

Conclusion

LC-MS/MS based analysis of tryptic and chymotryptic peptides of samples matched with the known amino acid sequence of Erythropoietin and provided positive confirmation of presence of Erythropoietin.

Isoelectric Focusing (IEF)

Methodology

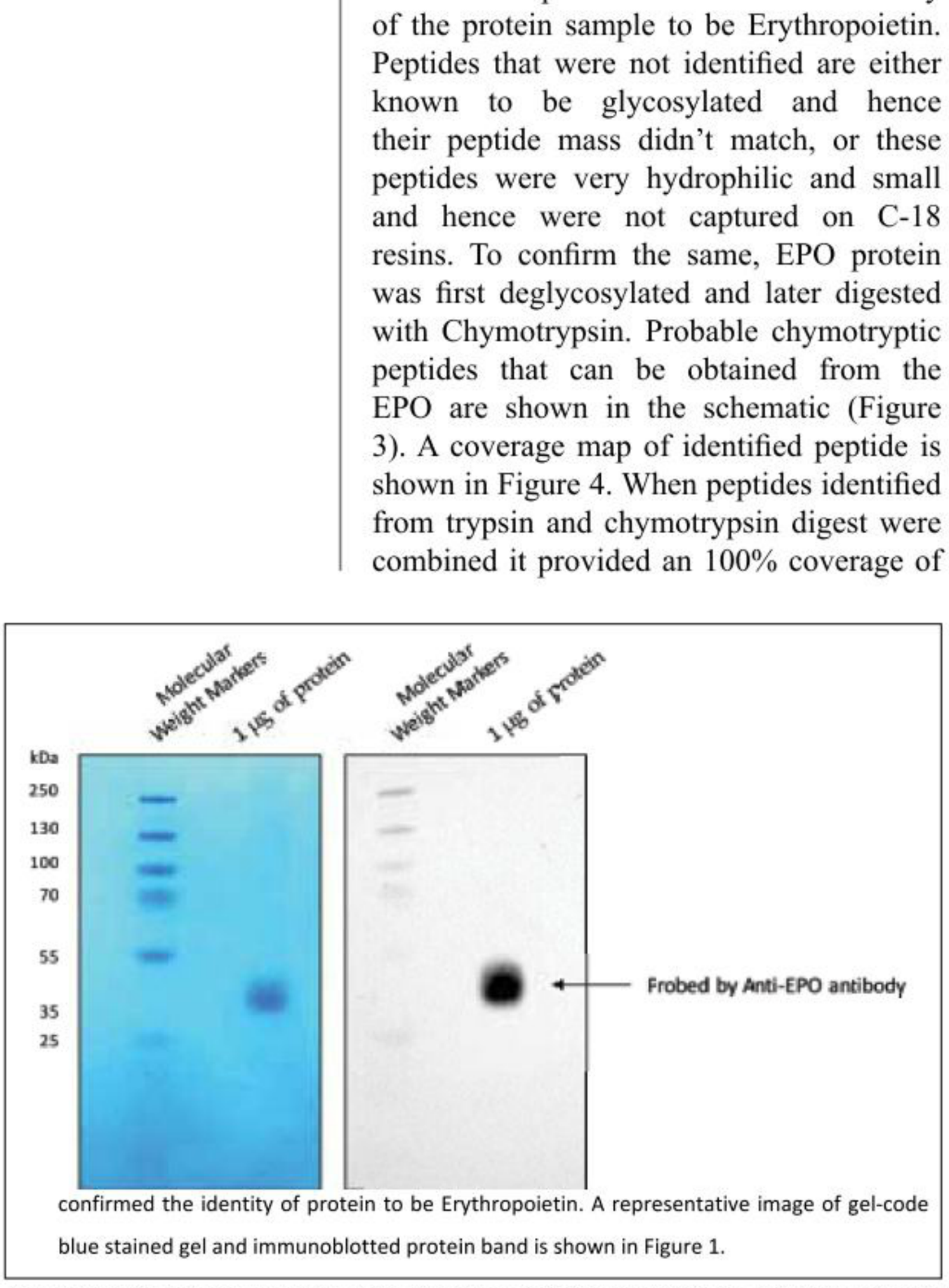
Erythropoietin Concentrated Solution was desalted by subjecting the sample to ultra-filtration using a 3 KDa cut-off filter. 10 µg of sample was then dissolved in IEF solubilization buffer containing 8M Urea, 30mM CHAPS, 10% glycerol. Sample was then reduced using 1% DTT for 30 min at 56° Centigrade. 10 µg of the EPO test sample were electrophoresed on a 4% poly-acrylamide gel containing 8 M Urea, 2% CHAPS and 2% ampholyte (pI range 3-5). After completion of the run, proteins in the gel were stained with gel code blue stain and de-stained with water. Gel images were captured using gel documentation system. A densitometry analysis of the gel image was performed to calculate the percentage contribution of each identified isoform.

Results, Interpretations and Discussions

Five isoforms of EPO were clearly identified in the pI range of 4 to 5. Proteins as visualized using gel-code blue stain is shown in Figure 5. Percentage contribution of each band was estimated using densitometry analysis and the results are listed in Table 2.

Conclusion

Isoelectric focusing identified five isoforms of Erythropoietin. The percentage ratio of isoforms present in the sample agrees with the known percentage ratio.



confirmed the identity of protein to be Erythropoietin. A representative image of gel-code blue stained gel and immunoblotted protein band is shown in Figure 1.

Fig. 1: Protein stained with Gel-Code Blue Stain (Left) and Probed by Anti-EPO antibody (Right).

Aggregate Analysis using HPLC-SEC

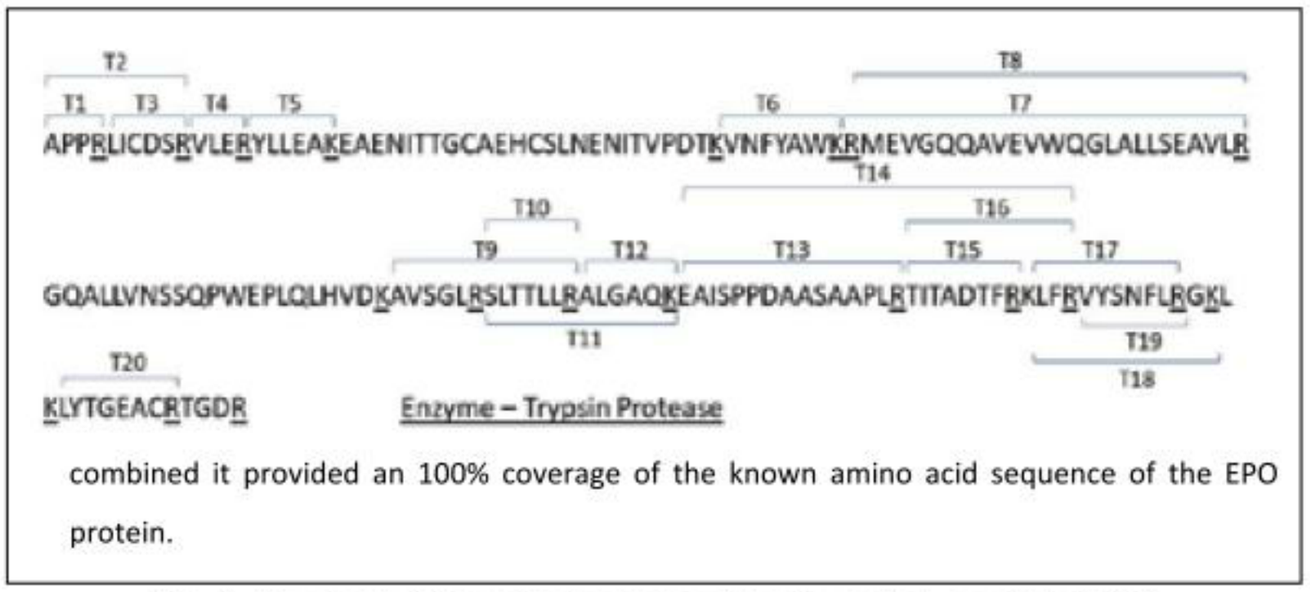
Methodology

Aggregate analysis was carried as per the guidelines provided in Indian Pharmacopeia. Briefly, EPO-Test sample at 20 micrograms and its 2% solution at 0.4 micrograms was prepared in mobile phase. Mobile phase was used as the Blank sample. Each sample was analyzed in duplicate. HPLC-SEC was performed using Bio SEC-5, 300Å, 7.8 x 300 mm, 5 µm, HPLC Chromatographic column with 1.5 mM Potassium Dihydrogen Phosphate, 8.1 mM Disodium hydrogen phosphate and 0.4 M NaCl (pH 7.4) as mobile phase. 100 µl of sample volume was injected on the column. The column temperature was maintained at 25˚C. Total run time was 30 min with flow rate of 0.5 ml/min. The chromatographic eluates were detected at 214 nm.

Results, Interpretations and Discussions

Primary chromatographic peak of the sample was found to be centered at 17.7 minutes (Figure 6). Chromatographic peaks that appeared before the retention time of the monomeric species of protein were considered as 'Aggregates'. Two minor aggregate peaks were observed at 11.4 and 16.2 minutes (Figure 7). Area under the curve (AUC) of all the identified chromatographic was calculated from background subtracted chromatogram and used in defining the percentage of aggregates and monomer in the sample (Table 3). Further, a 2% solution (equivalent to 0.4 µg of protein) of Test-Solution was subjected to similar analysis. With 2% test-solution load only a single chromatographic peak, at retention time 17.7 minute, was observed (Figure 6 and Figure 7).

As defined by Indian Pharmacopeia when AUC of individual or sum of aggregate peaks of 100% solution was compared with the AUC of principal peak of 2% solution, it was found to be significantly lower (>90%) concluding that the Aggregates are not present in the sample (Table 3).



combined it provided an 100% coverage of the known amino acid sequence of the EPO protein.

Fig. 2: Tryptic Peptides obtained after digestion of glycosylated EPO

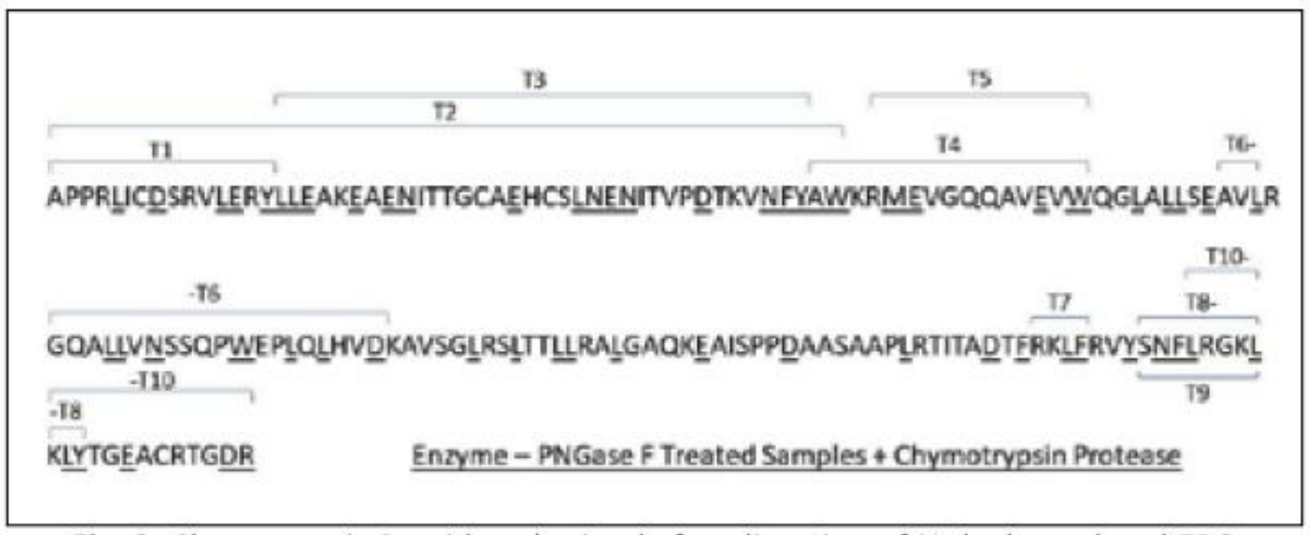


Fig. 3: Chymotryptic Peptides obtained after digestion of N-deglycosylated EPO

APPRLICDSRVLERYLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGL
ALLSEAVLRGQALLVNSSQPWEPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITAD
TFRKLFRVYSNFLRGKLKLYTGEACRTGDR

Fig. 4: A 100% coverage of known Amino Acid of EPO was confirmed through LC-MS/MS analysis

Conclusion

Aggregate analysis using HPLC-SEC method concluded that > 99.8 % of the sample is present in monomeric form.

Measurement of Sialic Acid Content

Methodology

Different dilutions of sialic acid (N-Acetylneuramic Acid) standard were prepared in the range of 40-200 µg/ml and the Test EPO sample was used at a concentration of 0.3 mg/ml. Equal volume of the Sialic acid standard and Test samples were taken separately in different glass vials. 1.0 ml resorcinol reagent was added to each tube and tubes were tightly closed and incubated in hot air oven at 100° C for 30 min. Content in each tube

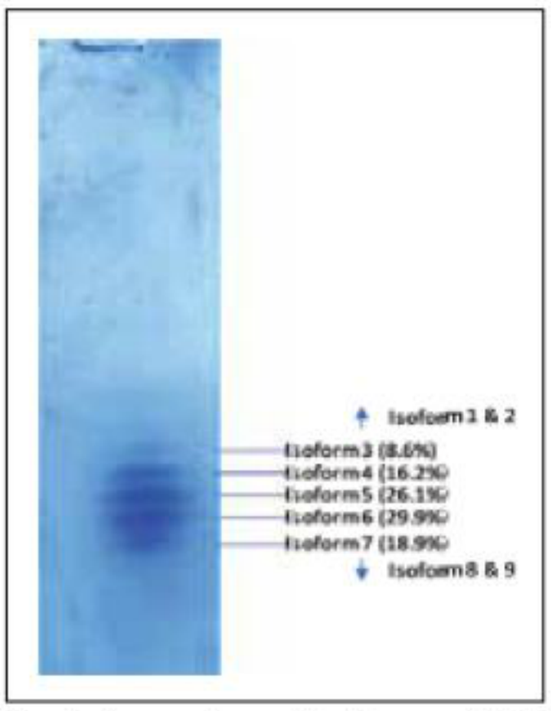


Fig. 5: Separation of Isoforms of EPO using Isoelectric Focusing

S. No.	Isoform Number	Content (%) as per IP	Content % as identified in this analysis
1.	1	0 – 15	ND
2.	2	0 – 15	ND
3.	3	1 – 20	8.6%
4.	4	10 – 35	16.2%
5.	5	15 – 40	26.1%
6.	6	10 – 35	29.9%
7.	7	5 – 25	18.9%
8.	8	0 – 15	ND

Table 2. *Estimation of percentage contribution of different isoforms using gel-IEF based method*

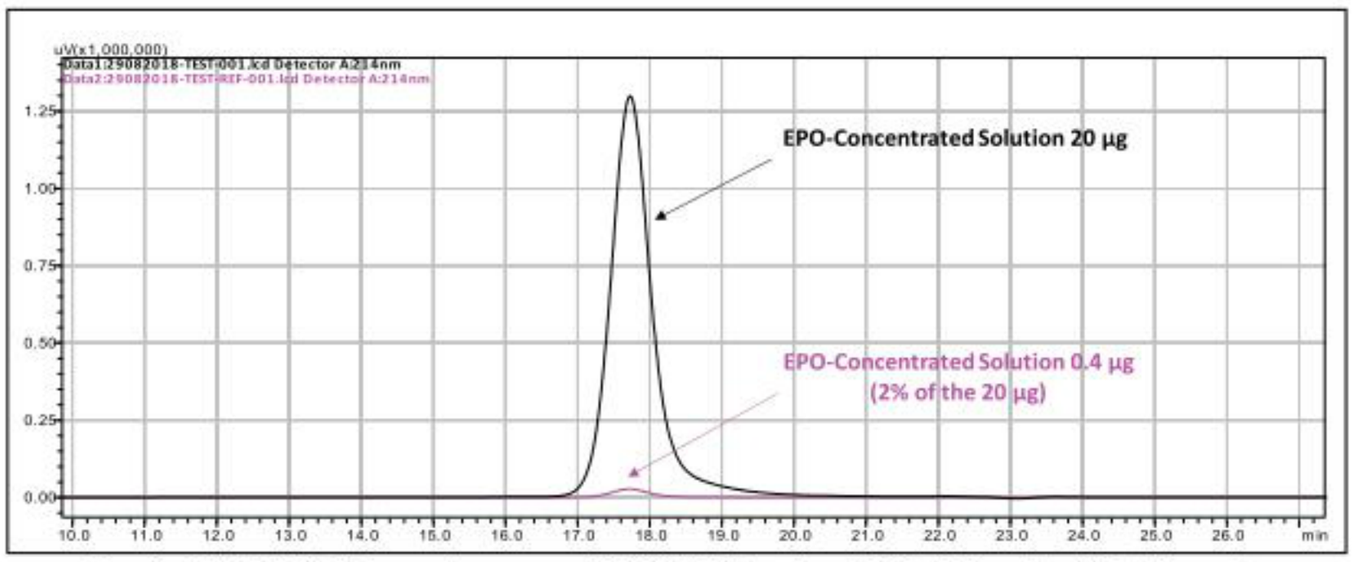


Fig. 6: HPLC-SEC Chromatogram of 100% (20 µg) and 2% (0.4 µg) of Test Sample

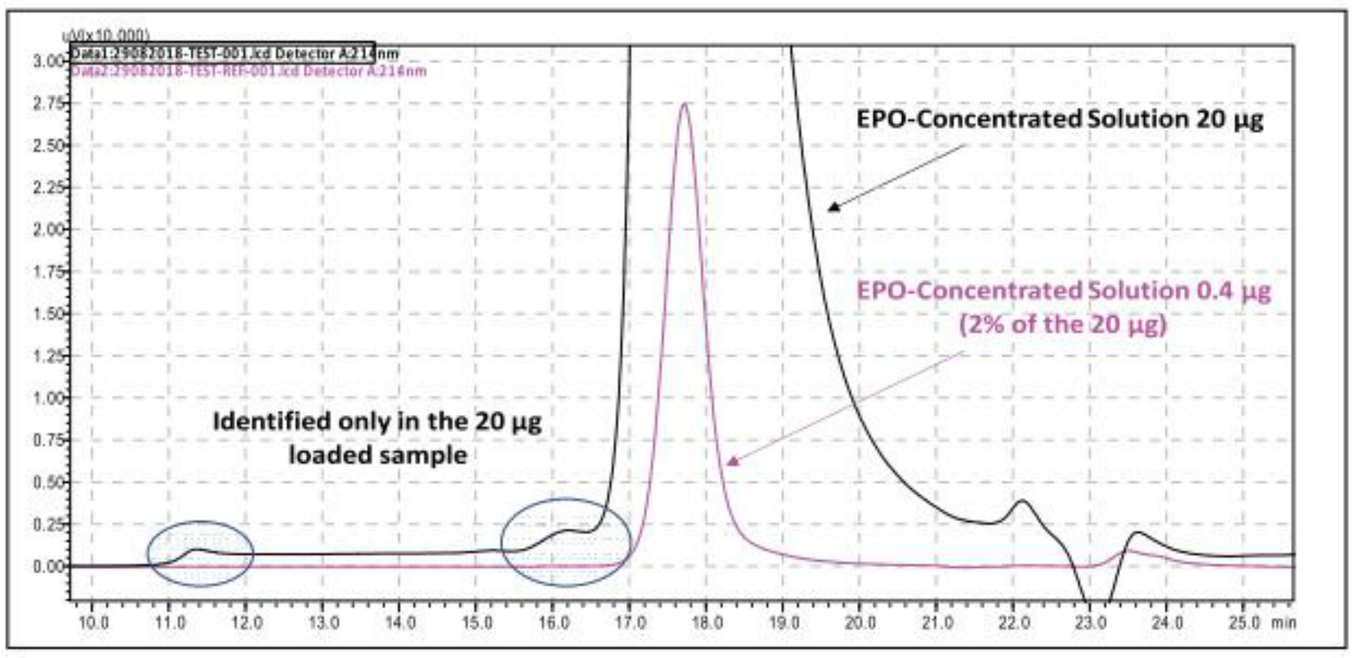


Fig. 7: Zoomed HPLC-SEC Chromatogram of 100% (20 µg) and 0.2% (0.4 µg) of Test Solution Showing Analysis Region

was cooled. Derivatized sialic acid were extracted using Butanol: Butyl acetate (12:48 v/v). Aqueous phase was carefully removed and its absorbance at 580 nm was measured. A standard concentration curve was generated using absorption values obtained from sialic acid standard. Absorbance of test EPO was determined from the sialic acid standard concentration curve using linear regression analysis. Sialic acids per mole of Erythropoietin was measured assuming that the relative molecular mass of EPO is 30600 Dalton and that the relative molecular mass of Sialic acid is 309 Dalton.

Results, Interpretations and Discussions

The standard concentration curve of Sialic acid is shown in Figure 8. The Sialic acid content in the Test solution was estimated to be 20.2 ± 1.7 moles per mole of EPO (Table 4).

Conclusion

Sialic acid content was estimated to be 20.2 ± 1.7 moles per mole of Erythropoietin.

N-Glycan Profiling using LC Fluorescence

Methodology

80 µg of the test sample was precipitated using acetone and deglycosylated by treating it with PNGase-F enzyme through 24 hours of incubation at 37 °C. N-glycans were extracted by treating the sample with ice cold ethanol. Extracted N-glycans were labelled with 2-AB through Sodium Cyanoborohydride treatment. Excess of 2-AB was removed using liquid-liquid extraction with chloroform: water (1:1 v/v). Clarified 2-AB labelled glycans were lyophilized and stored at -20 °C till further analysis. The N-Glycans were separated using HPLC-HILIC (hydrophilic interaction liquid chromatography) method. A 30 µl of sample was injected on to ZORBAX-RRHD-300-HILIC column (2.1 X 100 mm). N-glycans were separated using a gradient of Mobile Phase A: 50 mM Ammonium Format and Mobile Phase B: 100% Acetonitrile, ranging from 90% to 60% of mobile phase B, developed over 50 minutes. Eluates were detected through an online fluorescence detector using excitation wavelength of 330 nm and emission wavelength of 420 nm.

Results, Interpretations and Discussions

Based on the hydrophilicity of FA2G2 (Di-antennary), FA3G3 (tri-antennary) and FA4G4 (tetra-antennary) glycans species, FA2G2 glycans were eluted early and they were followed by FA3G3 and FA4G4 glycan species (Figure 9). Peaks were assigned to FA2G2, FA3G3 and FA4G4 based on the reference literature (Anumula KR 2006) and mass data obtained from the fractions (data not shown). Area under the curve of the separated bi, tri and tetra sialic glycans can be used to calculate the 'hypothetical N-glycan charge Z' (Hermentin *et al*. 1996) that can characterize protein glycosylation in a simple, however efficient manner.

Conclusion

It was reconfirmed that N-Glycans of EPO contains Bi-Antennary, Tri-Antennary and Tetra-Antennary glycans.

O-Glycan Analysis using LC-MS

Methodology

O-glycan analysis was performed while the O-glycans were still linked to the proteins. Pellet fraction obtained after the extraction of N-Glycan was taken and passed through a C4 resin HPLC-CHIP and elutes were infused into an Q-TOF mass-spectrometer over a period of 10 minutes. The mass over charge spectra of the sample were obtained. The charges in the spectra were deconvoluted using MassHunterBioConfirm software which uses Peak Modelling Charge Deconvolution (pMod), an enhanced deconvolution algorithm based on Maximum Entropy deconvolution.

Results, Interpretations and Discussions

The charged spectra were analyzed for protein mass and alteration in protein mass due to presence of O-glycan. Online ESI-MS provided detailed information of O-linked glycan (Figure 10). The mass of three most abundant LC peaks were deconvoluted to be 18239.40, 18896.8 and 19187.31 Da. 18239.40 Da was attributed to EPO in Aglycosylated protein form in reduced state with C-terminal arginine truncation. The observed additional mass of 656.73 Da was assigned to O-linked trisaccharide species with 1 hexose, 1 N-acetylhexosamine, and 1 N-acetylneuraminic acid. A further addition of mass of 291.19 Da is indicative of presence of additional N-acetyl neuraminic acid. Thus, mass of 18896.8 and 19187.31 Da were assigned to and tri and tetra saccharide O-linked glycans species of EPO, respectively.

Conclusion

There are no enzymatic digestion-based methods available for the analysis of

Analysis of Chromatograms — Analysis as per Indian Pharmacopeia

Test (Replicate-1)

Peak#	Retention Time	Area	Conc.	Area%	Assigned as
1	11.4	17645	0.0	0.0	Aggregate
2	16.2	81809	0.2	0.2	Aggregate
3	17.7	54305720	99.8	99.8	Monomer
Total		54405174	100.0	100.0	

TEST (Replicate-1)

Peak#	AUC of Peaks before Principal Peak
1	17645
2	81809

Test (Replicate-2)

Peak#	Retention Time	Area	Conc.	Area%	Assigned as
1	11.4	17297	0.0	0.0	Aggregate
2	16.2	86778	0.2	0.2	Aggregate
3	17.7	54326143	99.8	99.8	Monomer
Total		54430218	100.0	100.0	

TEST (Replicate-2)

Peak#	AUC of Peaks before Principal Peak
1	17297
2	86778

Test-2% Replicate-1

Peak#	Retention Time	Area	Conc.	Area%	Assigned as
1	17.7	1087249	100.0	100.0	Monomer
Total		1087249	100.0	100.0	

TEST-2% (Replicate-1)

Peak#	AUC of Principal Peak
1	1087249.0

Test-2% Replicate-2

Peak#	Retention Time	Area	Conc.	Area%	Assigned as
1	17.7	1196278	100.0	100.0	Monomer
Total		1196278	100.0	100.0	

TEST-2% (Replicate-2)

Peak#	AUC of Principal Peak
1	1196278.0

Total Area of any peak eluted before the Principal Peak is not more than the area of Principal Peak in the chromatogram obtained with 2% of the Test-Solution.

Table 3. *Analysis of HPLC-SEC Chromatograms*

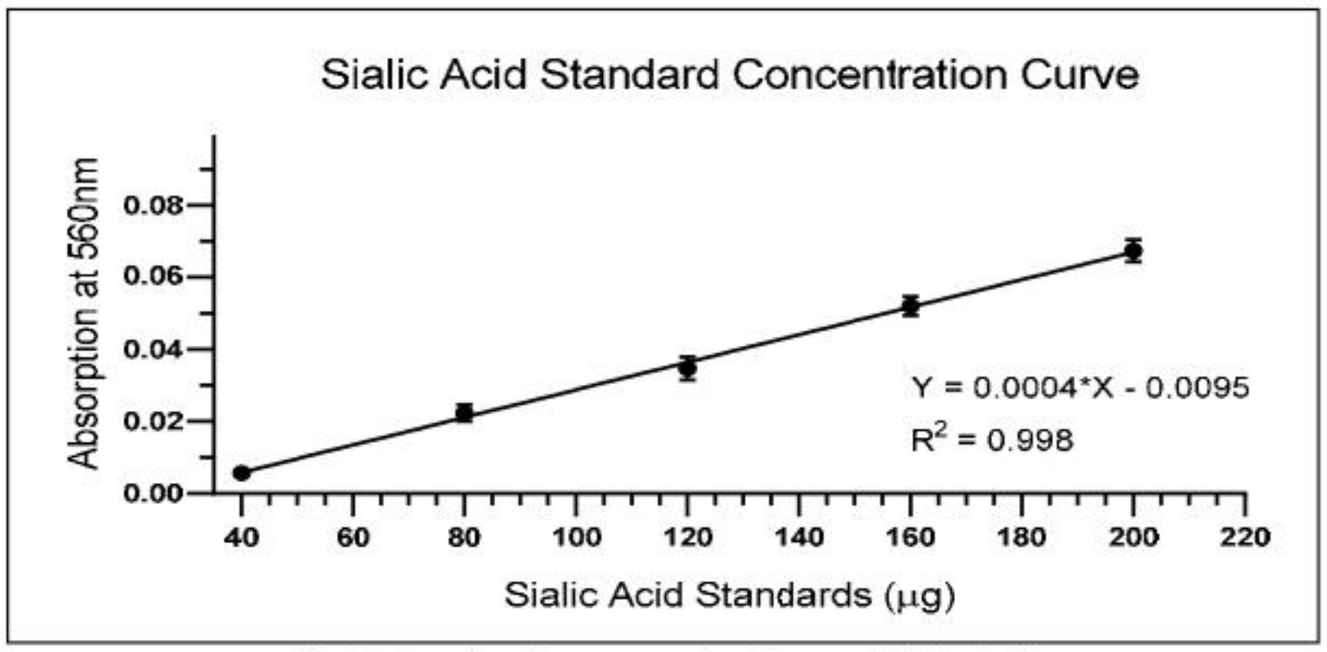


Fig. 8: Standard Concentration Curve of Sialic Acid

Sr. No.	Sample	Moles of Sialic Acid per Moles of EPO			
		Replicate -1	Replicate -2	Replicate -3	Average ± SD
1.	Test-Solution	18.57	20.22	21.87	20.2 ± 1.7

Table 4. *Estimated Sialic Acid Content of EPO*

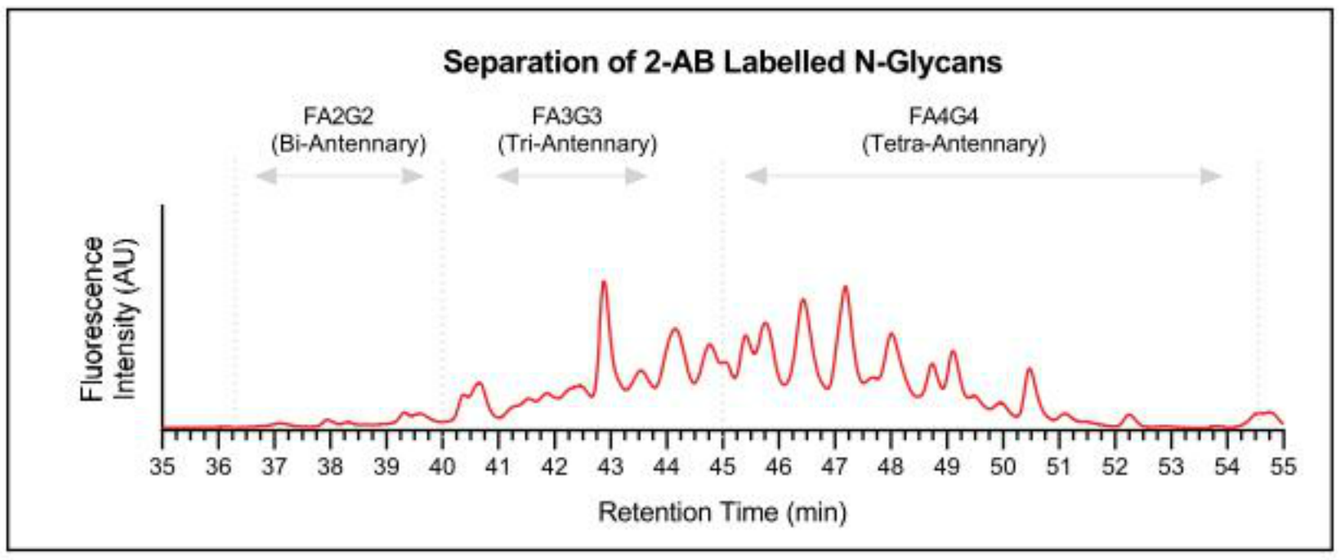


Fig. 9: N-glycan Profile of EPO Products

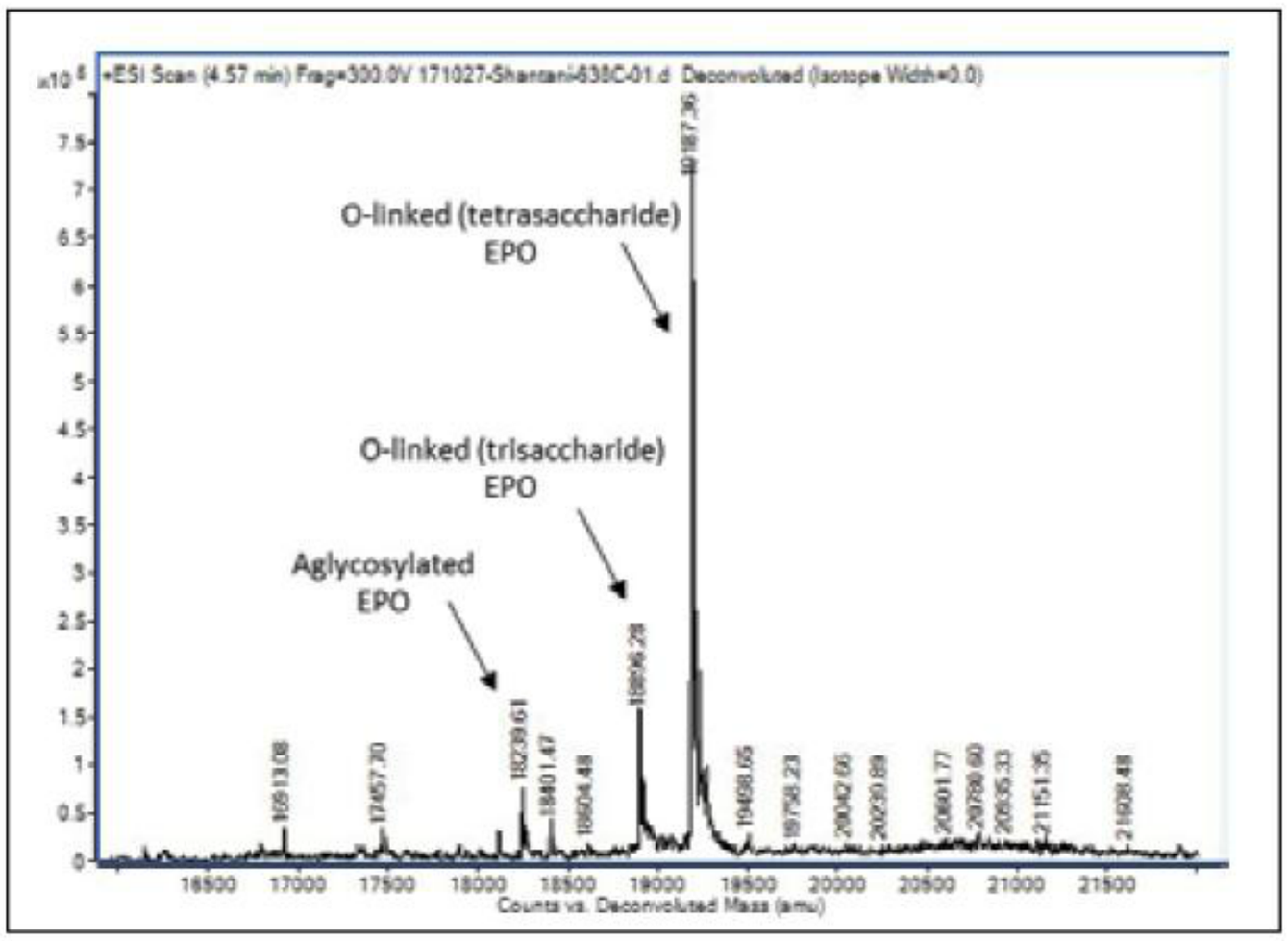


Fig. 10: LC-MS profile of O-linked glycans in EPO Sample

O-Glycans. On column O-glycan analysis carried out here using LC-MS based approach clearly identified at least two O-glycoforms in EPO sample.

Study Conclusion

The identity, integrity and purity of the EPO concentrated sample was confirmed after analyzing the given sample by chromatographic, spectroscopic, immunoblotting and mass-spectrometry based methods.

Shantani's Strength

Shantani carry out experiments following the ICH-Q6B guidelines under a Quality Management System that confers to ISO 17025:2017. A team of dedicated, specialized protein scientists utilize their decades old expertise in analyzing data obtained from different techniques in making appropriate conclusions.

Acknowledgement

We would like to thank the management of Shantani Proteome Analytics Pvt. Ltd. for their support and encouragement. Special thanks to Mr. Shrikant Mane and Ms. Sneha Pise for their contribution in the method development work. We would also like to express our gratitude to the entire analytical R&D team of Shantani for their kind assistance.

References

1. *Anumula KR, Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. Analytical Biochemistry, 350(1):1-23. (2006).*

2. *Hermentin et al. 1996, The hypothetical N-glycan charge: a number that characterizes protein glycosylation. Glycobiology, Volume 6, Issue 2, Pages 217–230 (1996).*