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Unraveling proteoform complexity by native liquid chromatography-mass spectrometry

Schaick, G. van

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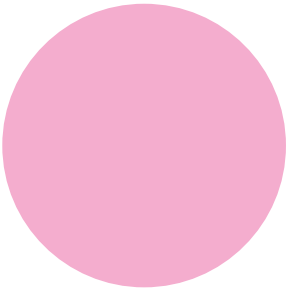

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Anion exchange chromatography - mass spectrometry for monitoring multiple quality attributes of erythropoietin biopharmaceuticals

G. van Schaick [1‡], C. Gstöttner [1‡], A. Büttner [2], D. Reusch [2], M. Wuhrer [1], and E. Domínguez-Vega [1]

[1] Center for Proteomics and Metabolomics, Leiden University Medical Center, Leiden, Netherlands.

[2] Pharma Technical Development Europe, Roche Diagnostics GmbH, Penzberg, Germany

[‡]Authors contributed equally to this work

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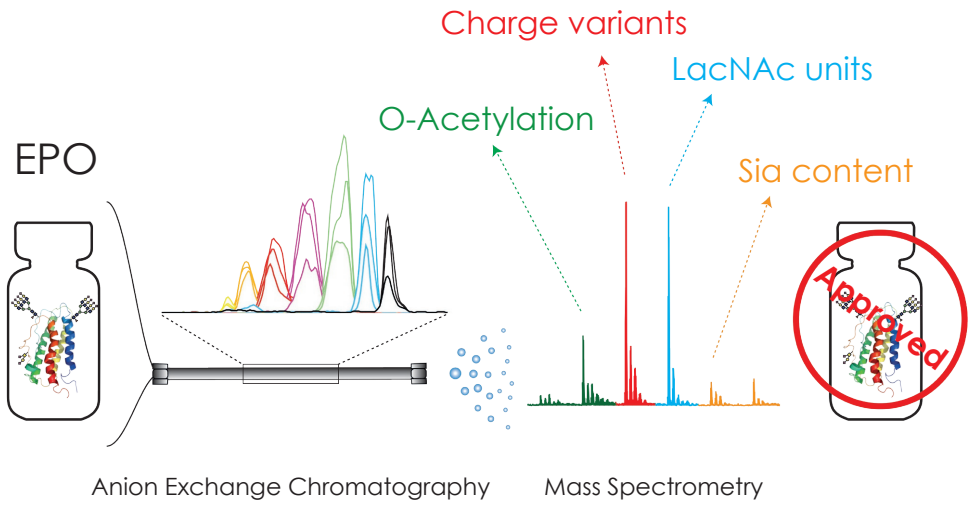
Assessment of critical quality attributes of the biopharmaceutical erythropoietin (EPO) prior to product release requires the use of several analytical methods. We developed an MS-compatible anion exchange (AEX) method for monitoring multiple quality attributes of EPO biopharmaceuticals. AEX was performed using a stationary phase with quaternary ammonium functional groups and a pH gradient for elution. Separation of charge variants and high-quality MS data were achieved using 30 mM ammonium formate pH 5.5 and 30 mM formic acid pH 2.5 as mobile phases. In a single experiment, assessment of critical quality attributes, such as charge heterogeneity, sialic acid content and number of N-acetyllactosamine units, was possible while providing additional information on other modifications such as O-acetylation and deamidation. In addition, good repeatability and robustness for the relative areas of the individual glycoforms and average number of Neu5Ac per EPO molecule were observed. The results were comparable to common pharmacopeia and standard methods with the advantage of requiring fewer analytical methods and less sample treatment saving time and costs.



4.1 Introduction

Erythropoietin (EPO) is a hormone which, in its recombinant form, is used for the treatment of anemia [3]. Currently, more than 10 companies have EPO biologics, derivatives or biosimilars in their portfolio with totaled sales exceeding 20 billion USD [231]. EPO contains three N- and one O-glycosylation sites, with their glycans contributing to about 40% of its molecular mass [232]. EPO can be highly sialylated with up to 14 Neu5Ac distributed over three tetraantennary N-glycan structures and one disialylated O-glycan. Moreover, EPO variants carrying up to 15 sialic acids, with one tetraantennary glycan carrying five Neu5Ac, have been reported [233]. The glycosylation of EPO is important for its stability, folding and secretion [234, 235], can influence its immunogenicity [236], and plays a crucial role for the *in vivo* biological activity [237]. More specifically, a high number of sialic acids (Sia) relates to an increased *in vivo* bioactivity and half-life of EPO [37]. Another factor influencing the half-life can be the number of N-acetyllactosamine (LacNAc) units [38]. These modifications together with other parameters (e.g., charge variant distribution) are considered critical quality attributes (CQAs) and should be within certain ranges for product release. Other modifications such as O-acetylation of Neu5Ac or deamidation of asparagine residues may also appear and potentially affect half-life [54] or biological activity [238, 239], respectively.

To monitor all these aspects a range of different quality control (QC) methods is required. For charge heterogeneity, the standard method described in pharmacopeia relies on the separation of charge variants of EPO (i.e., sialylation and deamidation) by capillary electrophoresis and UV detection (CE-UV) [240]. It has been suggested that the relative peak area of each separated peak (charge variant) can be used to predict EPO potency and it is currently under evaluation for replacement of the *in vivo* bioassay [241]. This method, however, is not mass spectrometry (MS)-compatible and does not provide information on other attributes. Therefore, additional QC methods are required for the assessment of Neu5Ac content (analysis of released Neu5Ac) and for the LacNAc units (analysis of released desialylated glycans) [233], which only provide information on a single CQA. Bottom-up approaches are rising for multiple attribute monitoring but often fail to predict attributes, such as charge profiles and require labor-intensive sample treatment [233]. Intact mass analysis, by direct infusion via ESI-MS [242] or by reversed-phase liquid chromatography hyphenated with MS [243-245], featured a simple sample treatment but suffered from vast data complexity with overlapping species and - importantly - did not permit assessment of charge profiles. Additional attempts enhanced the charge variant analysis by replacing the non-volatile

background electrolyte with volatile salts allowing hyphenation of CE with MS, yet these approaches lack of user-friendliness and robustness.

Here, we present a robust method for monitoring of several EPO CQAs (i.e., charge variant distribution, Neu5Ac content, LacNAc units) as well as additional attributes (i.e., O-acetylation, site occupancy, deamidation) by analyzing the intact protein level without need of extensive sample treatment. This method has sufficient separation power to resolve EPO charge variants while permitting extensive characterization when coupled to MS. The separation is achieved using strong anion exchange (AEX) stationary phase and an MS-compatible pH gradient for elution. While the coupling of ion exchange chromatography (IEX) to MS has been often applied for antibody characterization using cation exchangers [102, 115, 153, 157, 163], for anion exchanger the literature is limited [155, 157]. In addition, its potential has never been explored for highly sialylated proteins like EPO. The low isoelectric point (pI) of glycosylated EPO (between 5.2 and 4.4) makes this protein suitable for separation with AEX. The results were comparable with the ones obtained with various pharmacopeia and QC methods and the benefits of the proposed method were demonstrated with different EPO variants.

4.2 Experimental section

4.2.1 Materials and samples

Ammonium formate (≥ 97%) was obtained from Fluka (Steinheim, Germany). Cesium iodide (99.999%) was purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (≥ 98%) was obtained from Riedel-De Haen (Seelze, Germany). Purelab ultra provided the deionized water (ELGA Labwater, Ede, the Netherlands). EPO-RS and EPO samples with altered glycan profiles (EPO-1 and EPO-2) were provided by Roche Diagnostics (Penzberg, Germany). EPO-1 and EPO-2 were obtained by separation of EPO drug substance material with reversed-phase liquid chromatography (RPLC) and collection of early (EPO-1) and late (EPO-2) elution fractions. The collected fractions were further purified with preparative anion exchange chromatography. Structural characterization of EPO-1 and EPO-2 was performed using bottom-up proteomics. A detailed description of the preparation and characterization of these samples is described by Buettner et al. [233]. Prior to analysis, all EPO samples were buffer exchanged in three rounds to ammonium formate (30 mM, pH 5.5) using Vivaspin 500 spin filters with 10 kDa cutoff (GE healthcare). The final concentration of the samples was 2 mg/ml.

4.2.2 Strong anion exchange chromatography

AEX analyses were performed on a biocompatible Ultimate3000 system (Thermo Fisher Scientific) equipped with a quaternary pump, autosampler, column thermostat, and variable wavelength detector. The pH was measured using a Thermo Scientific Ultimate 3000 PCM-3000 pH and conductivity monitor. A Proteomix AEX-NP5 guard column (10 x 2.0 mm, 5µm, Sepax) and Proteomix AEX-NP5 analytical column (250 x 2.1 mm, 5µm, Sepax) were used. The separation was accomplished with a linear gradient from 100% buffer A (30 mM ammonium formate, pH 5.5) to 100% buffer B (30 mM formic acid, pH 2.5) in 45 min. Thereafter, a cleaning step of 5 min at 100% B and conditioning for 20 min at 100% A were performed. The column temperature, UV-detection wavelength, and flow rate were 25°C, 280 nm, and 0.2 ml/min, respectively. In total, 10 µg protein was injected for AEX-UV and 30 µg protein for AEX-MS measurements. For AEX-MS analyses, the flow rate was reduced with a factor 5 via a flow-splitter prior to the electrospray ionization (ESI) source.

4.2.3 Fourier transform ion cyclotron resonance mass spectrometry

For the AEX-MS measurements, a Bruker 15T solariX FT-ICR-MS (Bruker, Daltonics, Bremen Germany) was used. Each measurement was started with calibration by direct infusion of cesium iodide (2.0 mg/ml in 50% acetonitrile + 0.1% formic acid). The ESI source was operated in positive mode with a capillary voltage of 4000 V and the endplate offset of -500 V. Nebulizer gas pressure, dry gas flow rate and dry gas temperature were 0.8 bar, 3.0 l/min, and 200 °C, respectively. The ion funnel 1 was operated at 180 V, radio frequency amplitude at 300 V_{pp} and skimmer 1 at 150 V. The in-source collision energy was 40 V. The collision voltage in the collision cell was set to -15 V. The used *m*/z-range was 1287.5-4000. Accumulation time was set to 1 s and the amount of data acquisition size to 1 M points. Each spectrum in serial mode analysis resulted from the summation of 5 spectra.

4.2.4 Data analysis

All MS data was processed in DataAnalysis (Bruker Daltonics). The raw spectra were deconvoluted using the Maximum Entropy tool with a mass range of 26000-34000 Da and an instrument resolving power of 60000. The SNAP algorithm (v2.0, Bruker Daltonics) was used to determine the mass of the monoisotopic peak after deconvolution (quality factor threshold is 0.5, signal-to-noise threshold is 2 and maximum charge state is 20). Chromatograms were smoothed using the Gauss smoothing algorithm (1 cycle). For calculation of the glycan compositions, monoisotopic masses were used, including hexose (Hex, 162.05 Da), N-acetylhexosamine (HexNAc, 203.08 Da),

Fucose (Fuc, 146.06 Da), N-Acetylneuraminic acid (Neu5Ac, 291.10 Da), N-glycolylneuraminic acid (Neu5Gc, 307.09 Da), acetylation (Acetyl, 42.01 Da), and phosphorylation (Phos, 79.97 Da). The relative abundance of the charge variants was calculated by integrating the peaks corresponding to each isoform in the total ion chromatogram and calculating their relative abundance. For the determination of the average number of Neu5Ac per EPO molecule, the extracted ion chromatograms (EICs) of the different sialylated forms were generated by extracting the most intense ion for each isoform followed by integration of the area under the peak and determination of their relative abundance. The average number of Neu5Ac molecules per EPO molecule was calculated by multiplying the relative abundance of each specie by the corresponding number of Neu5Ac and summing up the obtained values. For the determination of deamidation, for each species (non-deamidated and deamidated) the most intense ion was extracted with a window of ± 0.5 *m/z*. For co-eluting species, such as acetylated and different number of LacNAc units, the peak of interest was deconvoluted and the intensity of each species in the deconvoluted mass spectrum was used for the relative quantification.

4.3 Results and discussion

IEX separates proteins and proteoforms primarily on the basis of their pI [246]. The pI of EPO varies depending on the glycosylation. The theoretical pI of protein backbone is 7.9, however, adding glycosylation decreases the theoretical pI to 5.2-4.4 (i.e., higher number of Sia results in a lower pI). The separation of these variants with different pI values was achieved using an AEX column with quaternary ammonium functional groups as stationary phase and a pH gradient for elution (from slightly acidic to low pH). A column with non-porous particles and a particle size of 5 µm was chosen to obtain high separation efficiency. Column particles consisted of highly cross-linked poly(styrene divinylbenzene) (PS/DVB) with a hydrophilic layer capped with quaternary ammonium functional groups resulting in higher capacity compared to conventional non-porous particles. Injection of 50 µg protein did not result in loading capacity problems. Additionally, these particles are known to have sufficient stability at low pH [82]. We aimed to develop an MS-compatible method, therefore, different volatile salts (i.e., ammonium acetate and formate) were evaluated for the mobile phase. Using ammonium acetate highly sialylated species did not elute while ammonium formate permitted elution of all the species. Moreover, various salt concentrations (≤ 50 mM) of ammonium formate were tested (**Fig. S1**). Variation of the salt concentration between 20-40 mM did not affect significantly the EPO profile. Increasing the

concentration to 50 mM resulted in lower separation quality, especially for the early eluting peaks. The use of 30 mM ammonium formate pH 5.5 (A) and 30 mM formic acid pH 2.5 (B) provided optimal separation of charge variants as well as high-quality MS data. Using these mobile phases, a broad gradient from 0 to 100% B was found to be optimal for the separation of EPO proteoforms. The pH during the separation was monitored with an inline pH meter showing a linear decrease in pH (**Fig. S2**). The MS detection with a 15T Fourier transform ion cyclotron resonance-MS (FT-ICR-MS) instrument provided isotopic resolution, enabling the determination of the monoisotopic mass with high mass accuracy. The observed charge state distribution ($[M+8H]^{8+}$ to $[M+11H]^{11+}$) is consistent with previous native MS analyses of EPO [242] suggesting that the conditions during the separation and MS detection do not significantly alter the structure of the protein.

In-depth characterization of proteoforms of EPO reference standard (EPO-RS) was performed using the newly developed AEX-MS method. The separation of EPO-RS resulted in several peaks corresponding to the charge variants (**Fig. 1** and **S3**). The obtained profile is comparable to the profile of the CE-UV method based on the pharmacopoeia [240] with very similar charge variant distribution (**Fig. 1**; **Table S1**). Hyphenation with MS permitted further identification of the charged variants. Proteoforms were assigned based on the calculated monoisotopic mass of the protein containing different glycans attached. The assigned proteoforms were in agreement with previous studies [38, 233]. Sialylated forms were the main contributor to the charge heterogeneity with variants containing different number of Neu5Ac per EPO molecule (ranging from 8 to 14 Neu5Ac). Deamidation was also identified as a contributor to the charge variant profile and was detected with high confidence based on retention time shift and the isotopic distribution (**Fig. S4**; **Table S4**). The average number of Neu5Ac per EPO molecule obtained with the AEX-MS method (11.6 mol Neu5Ac/mol EPO) was in line with the value obtained by the release method (11.3 mol Neu5Ac/mol EPO, acceptance criterion ≥10.4) (**Table S2**). Within each peak, the deconvoluted mass spectra revealed multiple MS signals differing by a mass of 365.1 Da corresponding to a different number of LacNAc units (**inset Fig. 1**; **Table S4**). Addition of LacNAc units does not alter the charge (i.e., pI) of the protein and resulting variants were not separated with AEX. This permitted clustering of masses from EPO glycoforms with the same number of Sia but different number of LacNAc units allowing easy comparison between EPO products. Additionally, modifications of the Sia were detected (i.e., Neu5Gc instead of Neu5Ac (+16.0 Da) and O-acetylation (+42.0 Da)). The portion of non-acetylated EPO was 68.2% and mono, doubly and triply O-acetylated glycoform compositions were assigned (**Fig. 4c**). Other less

abundant glycan features, such as phosphorylated high mannose N-glycans, were also observed. Overall, 357 different compositions were assigned with satisfying mass accuracy (**Table S4**). This number is considerably higher than previously reported (up to 236 compositions [242]) showing the benefit of the combination of the AEX separation with high-accuracy MS detection.

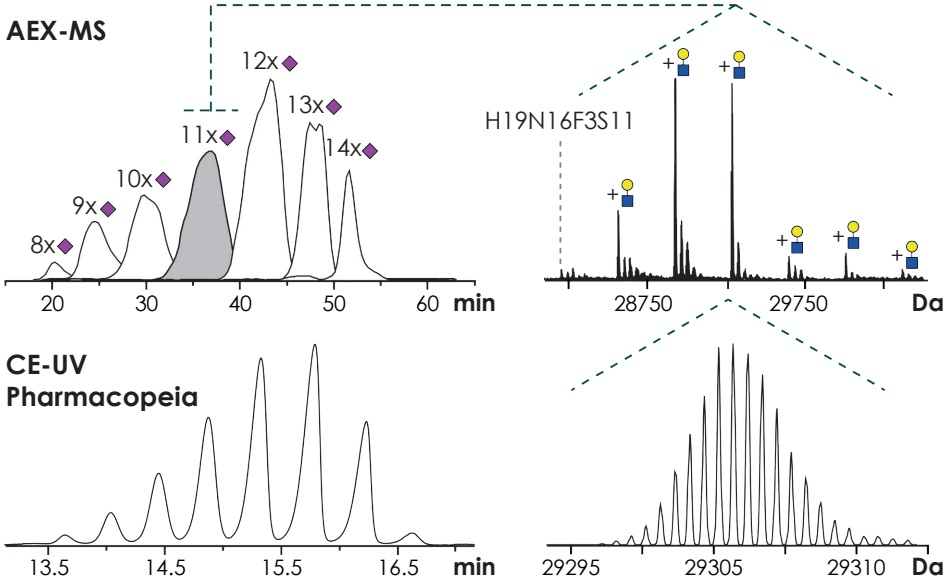


Figure 1 | AEX-MS of EPO RS shows high comparability with the pharmacopeia method for charge variants and allows further identification of the EPO proteoforms. Extracted Ion Chromatograms (EICs) obtained for EPO-RS and comparison with the CE-UV electropherogram obtained with the pharmacopeia method (lower trace). The number of Neu5Ac of each peak is indicated by ◆. The deconvoluted spectrum of the peak containing glycoforms with 11 Neu5Ac (grey fill) illustrate the differences in LacNAc units (■). Isotopic resolution was obtained, showed for glycoform H22N19F3S11 in the zoom between 29295 and 29315 Da. The UV chromatogram and the Base Peak Chromatogram can be found in **Fig. S3**.

Besides the ability to provide in-dept characterization of EPO-RS, the method must provide a repeatable and robust separation. To assess the repeatability, technical replicates of the EPO were injected over 1 week using the same sample and mobile phase. Low inter- and intraday variation was observed for the relative areas of individual glycoforms (SD 0.01 - 0.5%) and average number of Sia per EPO molecule (SD < 0.007) (**Table S1**) indicating good repeatability. Furthermore, additional measurements were performed after a longer period of time using new samples and mobile phases. The obtained values are similar to the repeatability results indicating good intermediate precision. The robustness was determined using a factorial randomized design with 4 factors. It was found that only the ionic strength of mobile phase B influenced the relative areas (**Fig. S5**; **Table S3**). Nevertheless, a change of 5

mM in mobile phase B concentration compared to the optimal method (i.e., 25 mM or 35 mM) still resulted in average numbers of Sia per EPO molecule above the acceptance criterion of 10.4. Other parameters (i.e., starting pH, salt concentration of mobile phase A and column temperature) had no significant effect demonstrating the robustness of the method.

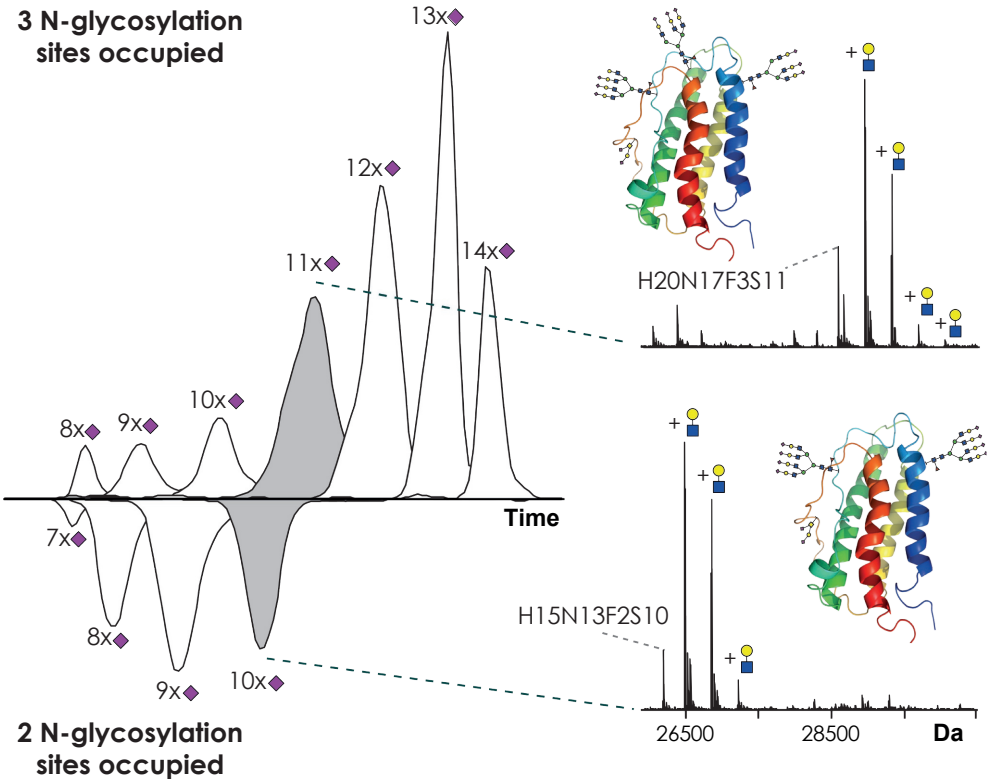


Figure 2 | AEX-MS of EPO-1 shows EPO variants with two and three N-glycosylation sites occupied. EICs of EPO-1 containing three N-glycans (upper part) or two N-glycans (lower part). Deconvoluted spectrum of the selected peaks are shown with LacNAc units indicated with ◆.

Two additional EPO variants with modified proteoform profiles (EPO-1 and EPO-2) were examined using the new AEX-MS method. In total, 313 and 290 different compositions were assigned for EPO-1 and EPO-2, respectively (**Table S5** and **S6**). EPO-1 exhibited a different profile than EPO-RS with a cluster of overlapping peaks at around 40 min. MS detection revealed EPO proteoforms carrying only 2 N-glycans (30.8%) overlapping with the proteoforms containing 3 N-glycans (69.2%; **Fig. 2**). The Neu5Ac profile of EPO-1 proteoforms showed a shift towards glycoforms with lower Neu5Ac content (average 11.0 mol Neu5Ac/mol EPO) compared to the EPO-RS (**Fig.**

4a and **b**). The percentage of acetylated Neu5Ac was also slightly lower (23.8%) compared to the EPO-RS (**Fig. 4c**). The elution profile of EPO-2 was very similar to EPO-RS with comparable number of Neu5Ac per EPO molecule (average 11.4 mol Neu5Ac/mol EPO) and acetylation levels (**Fig. 4b** and **c**). Only the isoforms containing 10, 11 and 12 Neu5Ac (*i.e.*, isoform 3, 4, and 5 in **Fig. 4a**) showed a slight increase. Despite the highly similar charge profile registered in UV, the MS data revealed a completely shifted pattern of LacNAc units (**Fig. 3** and **4d**). **Figure 3** shows the deconvoluted mass spectrum of the isoform with 11 Neu5Ac for RS and EPO-2, where the most intense glycoform of EPO-RS (H21N18F3S11) is shifted with 4 LacNAc units for EPO-2 (H25N22F3S11) highlighting the difference in LacNAc units and the simplicity of data comparison between EPO products. For both EPO variants the substitution of Neu5Ac with Neu5Gc was detected.

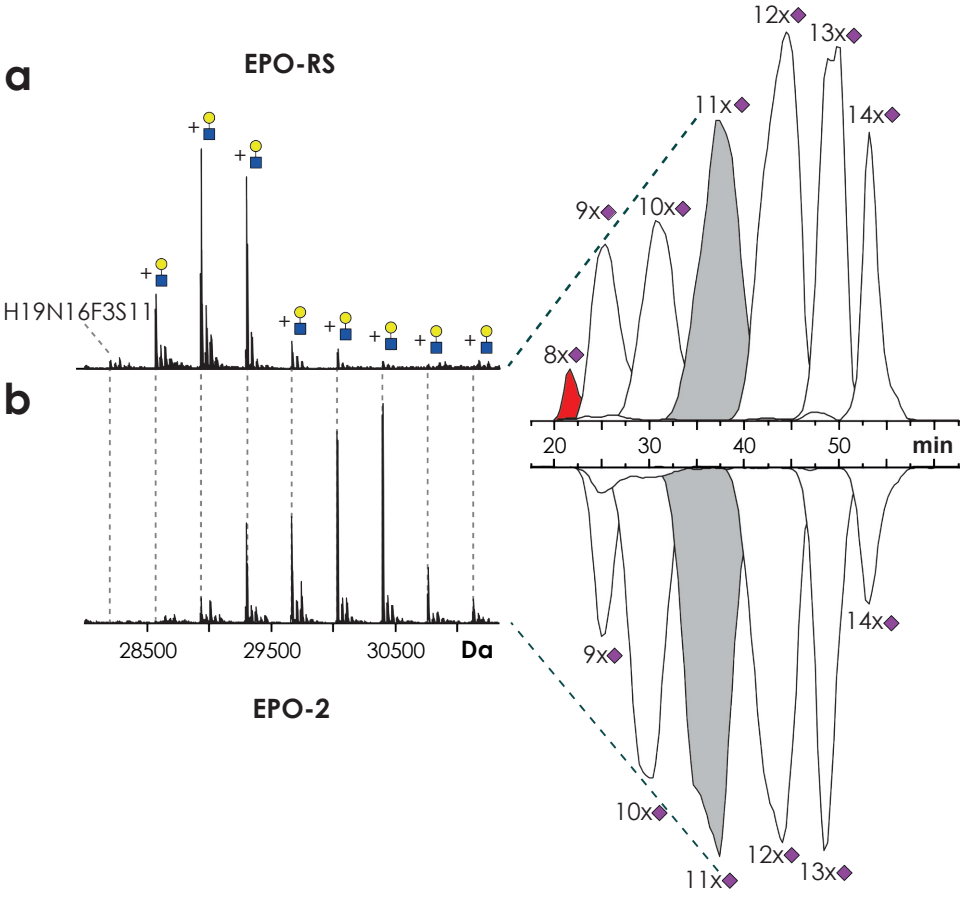


Figure 3 | Comparison of the AEX-MS analysis of EPO-RS (part a) and EPO-2 (part b) with deconvoluted MS spectra of the selected peaks. The LacNAc repeats are indicated by . Peak marked in red was only observed in the reference standard.

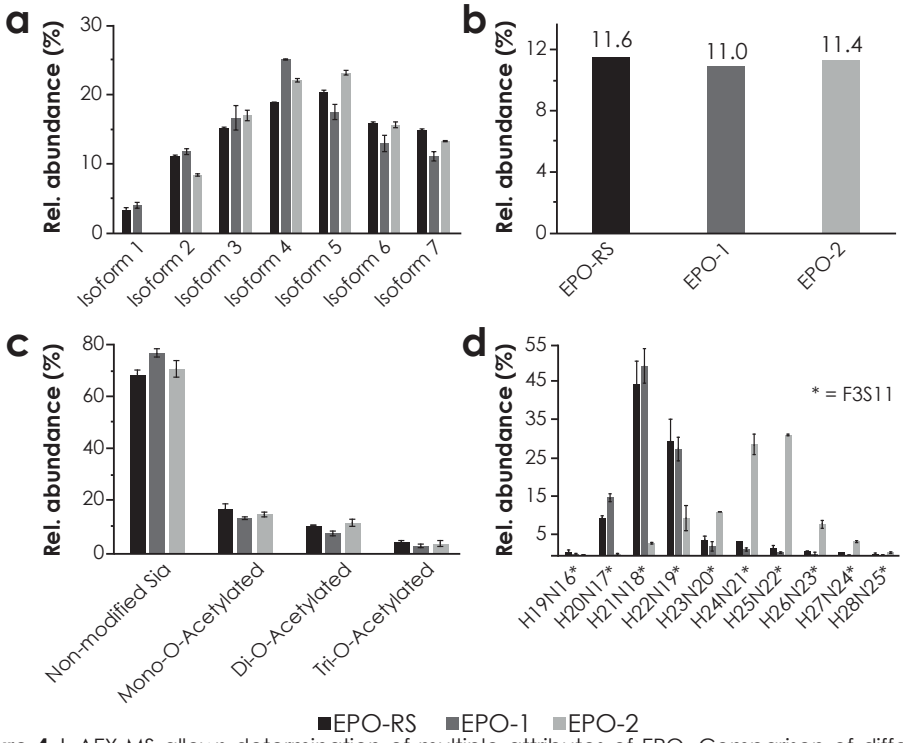


Figure 4 | AEX-MS allows determination of multiple attributes of EPO. Comparison of different attributes for EPO-RS, EPO-1 and EPO-2 after AEX-MS analysis. (**a**) relative amount of each charge variant, (**b**) average number of Neu5Ac per EPO molecule. (**c**) O-acetylation levels of Neu5Ac. (**d**) number LacNAc units and relative amounts. Data was analyzed in duplicate (technical replicates) and represents mean ± SD.

4.4 Conclusion

In summary, we propose a new method for simultaneously monitoring multiple EPO attributes based on AEX-MS separation using volatile buffers. Several CQAs (i.e., charge heterogeneity, Neu5Ac content and LacNAc units) and additional modifications (i.e., O-acetylation and deamidation) can be monitored in a single experiment. Comparison of the results with conventional methods revealed high similarity. Although the extended characterization of the EPO proteoforms may require dealing with complex data, monitoring of quality attributes can be performed in a straightforward manner. In particular, this robust method can be used with stand-alone UV detection in routine QC for the determination of the charge variants of EPO. Furthermore, EPO was maintained in a native-like state during separation opening possibilities for further fractionation and bioactivity assays on the resolved glycoforms. The proposed method enables EPO characterization at unrivaled level of detail with the potential for replacing several methods currently used for batch release while reducing hands-on time and costs.

Acknowledgments

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Supplementary information

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.11.027.

Optimization of the mobile phase salt concentration for the AEX separation of EPO-RS (**Fig. S1**); pH gradient overlay with EPO chromatogram and programed gradient (**Fig. S2**); Charge variant separation of EPO with AEX-UV and AEX-MS (**Fig. S3**); Confirmation of the presence of deamidation in EPO-RS sample (**Fig. S4**); Influence of different variables to the robustness of the AEX-MS method obtained by a factorial randomized design (**Fig. S5**).

Comparison of the charge variant profile of EPO-RS obtained by AEX-UV and the CE-UV pharmacopeia method (**Table S1**); Comparison of average number of Neu5Ac obtained for EPO-RS with AEX-MS and the QC method (**Table S2**); Factors and responses for the DoE (**Table S3**); Assignments of masses observed for the analysis of EPO-RS (**Table S4**), EPO-1 (**Table S5**), and EPO-2 (**Table S6**).

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