CHARACTERIZING A NOVEL HYPOSIALYLATED ERYTHROPOIETIN BY INTACT GLYCOPROTEIN AND GLYCAN ANALYSIS

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Abstract

NeuroEPO plus is a recently developed recombinant human erythropoietin (rhEPO) without erythropoietic activity and shorter plasma half-life due to its low sialic acid content. This novel rhEPO product is under investigation as therapeutic protein in the treatment of neurodegenerative diseases owing to its neuroprotective and neurodegenerative properties. In this study, an in-depth characterization of NeuroEPO plus N-glycans was performed by a glycan isotope [$^{12}C_6$]/[$^{13}C_6$] coded aniline labeling strategy followed by capillary zwitterionic hydrophilic interaction liquid chromatography-mass spectrometry (CapZIC-HILIC-MS). A superior amount of low sialylated glycans and less branched structures were detected in NeuroEPO plus compare to other commercial rhEPOs. At the intact glycoprotein level, NeuroEPO plus glycoforms were separated by capillary zone electrophoresis with ultraviolet detection (CE-UV), optimizing the composition and pH of the separation electrolyte. Moreover, an isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) method was also optimized for the simultaneous analysis of this basic rhEPO and conventional acidic rhEPO products. The proposed glycomic and intact glycoprotein methods provide a robust and reliable analytical platform for NeuroEPO plus characterization and for its future implementation as biopharmaceutical in neurodegenerative diseases.

Introduction

Human erythropoietin (hEPO) is a highly glycosylated hormone, mainly synthesised by the kidney in adults. This glycoprotein is responsible of stimulating red blood cell production in the bone marrow, increasing oxygen supply to tissues [1, 2], and it has been described to exert an important role in the nervous system as neurotrophic and neuroprotective agent [3]. Recombinant human erythropoietin (rhEPO) was firstly produced in mammalian cells using recombinant DNA technology in the 80s. Since then, rhEPO has been widely used in the treatment of certain forms of anaemia and represents one of the largest biopharmaceutical markets. In the last decades, growing evidence has accumulated on the neuronal and vascular protection of rhEPO in multiple *in vivo* and *in vitro* experimental models [4–6]. Nevertheless, clinical studies on humans failed as higher doses and prolonged application caused cerebral hemorrhage, thrombosis, hypertension and edema, among others [6, 7]. For this reason, many efforts have been made to modify rhEPO with the aim of developing a new biopharmaceutical with similar neuroprotective capabilities, but without the negative effects of an increase of the hematocrit levels and blood viscosity [8].

Sialic acids have effects in the pharmacokinetics of rhEPO. The higher sialic acid content the longer serum half-life, and consequently, a greater hematopoietic activity is achieved [9]. On the other hand, Masuda S. *et al.* reported that recombinant rat EPO produced by astrocyte cell lines showed lower sialic acid content and had a slightly higher neuroprotective effect than that produced by CHO cell lines [10]. In this regard, several authors pointed out the existence of other types of EPO receptors in the nervous system, associated with non-hematopoietic functions such as tissue protection and repair, which seem to show higher affinity for hyposialylated EPOs [11, 12]. Hence, the Center of Molecular Immunology (CIM, Havana, Cuba) obtained a rhEPO product, called NeuroEPO, with low sialic acid content. Preclinical studies suggested that this

glycoprotein exhibited neuroprotective properties without erythropoietic activity [13, 14]. In phase I and physician-led clinical studies, its safety was proven but discrete positive effects on the cognitive functions of patients with Parkinson's disease were observed [15, 16]. For that reason, recently, a new rhEPO, named NeuroEPO plus, was developed by the CIM [17]. Modifications in the fermentation and purification processes made the glycosylation of this protein differ from the former NeuroEPO [13], which improves its efficacy as neurotherapeutic agent [17]. This molecule is currently in phase II-III clinical trials in Parkinson's and Alzheimer's diseases with very promising results. Nevertheless, an in-detail characterization of NeuroEPO plus glycosylation is still required. Current regulatory guidelines suggest that distribution of the main glycan structures of a therapeutic glycoprotein should be determined, paying special attention to the levels of mannosylation, galactosylation, fucosylation and sialylation, to ensure manufacturing consistency and product safety [18, 19]. Moreover, a comprehensive analysis of the glycan composition would also permit to delve into the role of NeuroEPO plus in the molecular and cellular mechanisms of neuroprotection and neuroregeneration. Unlike fluorescence detection methods commonly used in pharmaceutical industry [18, 19], the analysis of labeled glycans by mass spectrometry in combination with a glycan reductive isotope labeling (GRIL) strategy provides several advantages. It allows an unambiguous glycan identification and also a reliable quantitation of the changes resulted in the glycan profile of the target glycoprotein, caused by different manufacturing conditions. Moreover, this strategy can be useful to compare the glycan mapping of the protein of interest with other biopharmaceuticals [20]. In this sense, the analysis of NeuroEPO plus N-glycans by GRIL using $[^{12}C_6]$/$[^{13}C_6]$ coded aniline followed by capillary zwitterionic hydrophilic interaction liquid chromatography-mass spectrometry (CapZIC-HILIC-MS) would enable to reliably compare its glycosylation profile with commercially available erythropoietic rhEPO

products, and also evaluate the presence of glycan isomers differing in branching and linkage positions [21, 22].

At the intact glycoprotein level, the establishment of analytical methods for the separation and detection of NeuroEPO plus glycoforms is also necessary in quality control and final product release. Since the European Pharmacopoeia (Eur.Ph.) method was proposed in 2002 [23, 24], capillary zone electrophoresis with ultraviolet detection (CE-UV) has been implemented by rhEPO manufacturers in process development, characterization and quality control release. However, this method has not been yet evaluated for the analysis of basic rhEPOs with lower sialic acid content. Moreover, although the CE-UV method was considered to show better reproducibility and robustness in comparison with the former Eur.Ph. isoelectric focusing (IEF) test [23], the development of an isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) method for the simultaneous separation of basic and acidic rhEPOs products would be of great interest for biopharmaceutical companies as complementary methodology.

The present study is focused on the identification and reliable quantitation of NeuroEPO plus N-glycans by glycan isotope [$^{12}C_6$]/[$^{13}C_6$] coded aniline labeling and CapZIC-HILIC-MS analysis. Moreover, CE-UV and IEF-PAGE are also investigated for the analysis of NeuroEPO plus intact glycoforms. Results obtained using both techniques are discussed in order to provide a reference glycoform profile of this novel rhEPO product and establish a robust analytical platform for its characterization and future application as biopharmaceutical in neurodegenerative diseases.

2 Materials and Methods

2.1 Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Acetonitrile, acetone, acetic acid (HAc, glacial), formic acid (FA 98%-100%), dimethyl sulfoxide (DMSO), sodium acetate (NaAc), sodium chloride and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Ammonium hydrogen carbonate (NH_4HCO_3), sodium phosphate dodecahydrate ($Na_3PO_4 \cdot 12H_2O$), sodium cyanoborohydride ($NaBH_3CN$), [$^{12}C_6$]-aniline ([$^{12}C_6$]AN), [$^{13}C_6$]-aniline ([$^{13}C_6$]AN), 2-mercaptoethanol (β-ME), sodium dodecyl sulfate (SDS), 1,4-diaminobutane (putrescine), N-tris-[hydroxymethyl]methylglycine (tricine), malonic acid and urea were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen chloride (HCl, 37%) was supplied by Panreac (Barcelona, Spain) and "NP-40 alternative" by Calbiochem (Darmstadt, Germany). LC-MS grade ammonium acetate (NH_4Ac) and acetonitrile (ACN) from Merck (Madrid, Spain), and water from Fluka (Madrid, Spain), were used for CapZIC-HILIC-MS analysis. Pharmalytes® IEF carrier ampholytes (pH gradients 3-10 and 2.5-5) were supplied by GE Healthcare Bio-Science (Uppsala, Sweden). Peptide-N-glycosidase F (PNGase F) was obtained from Roche Diagnostics (Basel, Switzerland). ESI low concentration (ESI-L) tuning mix was supplied by Agilent Technologies (Waldbronn, Germany) for the tuning and calibration of the oa-TOF mass spectrometer. Water with a conductivity value lower than 0.05 $\mu S \cdot cm^{-1}$ was obtained using a Milli-Q water purification system (Millipore, Molsheim, France).

2.2 Recombinant erythropoietin samples

rhEPO produced in CHO cell lines was provided by the European Pharmacopoeia as Chemical Reference Substance (CRS-batch 1). Each sample vial contained 100 µg

erythropoietin (EPO-CRS; a mixture of epoetin alpha and beta), 0.1 mg Tween 20, 30 mg trehalose, 3 mg arginine, 4.5 mg NaCl, and 3.5 mg Na_2HPO_4. The content of each vial was dissolved in Milli-Q water to obtain a 1 mg·mL^{-1} solution of rhEPO. Two rhEPOs produced in CHO cell lines were provided by the Center of Molecular Immunology: EPOCIM® (batch 1) and NeuroEPO plus (batches 1 and 2). EPOCIM vials contained 1.23 mg·mL^{-1} of rhEPO and 0.02% Tween 20 in citrate buffer at pH 6.9. NeuroEPO plus vials contained 2.15 mg·mL^{-1} of rhEPO and 0.02% Tween 20 in phosphate buffer at pH 6.3.

Excipients of low-molecular mass were removed from rhEPO samples (EPO-CRS, EPOCIM and NeuroEPO plus) by passage through a Microcon-10kDa Centrifugal Filter (Millipore, Molsheim, France) as described in [25]. The filter membrane was initially washed with Milli-Q water for 10 min in a centrifuge at 13000 g. Then, the sample was centrifuged for 10 min under the same centrifugal force. The residue was washed three times with an appropriate volume of Milli-Q water using the same centrifugation conditions. The filtrates from each of the four previous centrifugation steps were discarded. The residue was recovered from the cartridge by centrifugation upside down into a new vial (3 min at 1000 g) and sufficient Milli-Q water was added to adjust the rhEPO concentration to 1 mg·mL^{-1}. rhEPO samples were kept at 4ºC between workdays and stored at -20ºC when not in use for a long period of time. Centrifugations were performed in a Mikro 220R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany).

2.3 N-glycan analysis

Dried rhEPO samples were reduced with 0.5% β-ME in the presence of 0.5% of SDS in 50 mM Na_3PO_4 (pH 7.5) and heated at 100 ºC in a thermoshaker for 30 min. When samples were at room temperature, a volume of 50 mM Na_3PO_4 (pH 7.5) with 1% (v/v)

of NP-40 alternative was added to achieve a final concentration of 0.1% of SDS and β-ME in the samples. To release the *N*-glycans, 1 µL of PNGase F (1 U) solution was added and the mixture was carefully vortexed and incubated at 37 ºC for 18 h. Afterwards, 1 µL more of PNGase F was added and the incubation was continued for additional 18 h. Digestion was stopped by adding ~3 µL of FA and released *N*-glycans were purified by solid phase extraction (SPE) using Hypercarb cartridges (25 mg, 1 mL volume, Thermo Fisher Scientific). SPE cartridges were firstly conditioned and equilibrated with 1 mL of 60% ACN, 0.1% FA and with 2 mL of water, respectively. Digested sample was loaded to the SPE cartridge diluted in ~500 µL of water and then rinsed with 1 mL of water. Retained *N*-glycans were eluted with 600 µL of 60% ACN, 0.1% FA and the eluate was evaporated to dryness by Speed Vac. Dried *N*-glycans were stored at -20 ºC until its use. Glycan labeling was carried out by adding 10 µL of reaction mixture (0.35 M aniline and 1 M $NaCNBH_3$ in DMSO with 30% HAc) to the dried glycans and the mixture was incubated for 2 h at 70 ºC. NeuroEPO plus samples were labeled with [$^{12}C_6$]-aniline while EPOCIM were labeled with [$^{13}C_6$]-aniline. After incubation, samples were cooled to room temperature and labeled glycans were precipitated with acetone as described in [21]. Subsequently, equimolar mixtures of NeuroEPO plus and EPOCIM were prepared and evaporated to dryness by Speed Vac. Dried *N*-glycans were stored at -20 ºC until analysis.

Labeled glycan samples were analysed by CapLC-MS using a 1200 Series capillary liquid chromatography system coupled to a 6220 oa-TOF LC/MS mass spectrometer with an orthogonal dual-nebulizer ESI source (Agilent Technologies). A ZIC-HILIC column packed with 3.5 mm particles, 150 x 0.3 mm L_T x ID (SeQuant, Umeå, Sweden) was used for chromatographic separations. Experiments were performed at room temperature with gradient elution at a flow rate of 4 $\mu L \cdot min^{-1}$ and injecting 0.25 µL of each glycan sample (glycan concentration: ~100 $pmol \cdot \mu L^{-1}$). Eluting solvents were A: 1 mM NH_4Ac solution and B: acetonitrile. The following gradient conditions were used:

solvent B from 90% to 80% (within 5 min) and from 80% to 65% (within 20 min) as linear gradient, followed by cleaning and equilibration steps of B: 65%→50% (within 5 min), 50%→0% (within 5 min), 0% (over 15 min), 0%→90% (within 5 min) and 90% (over 10 min). The operational MS conditions were established in negative ion mode: capillary voltage -3500 V, drying gas (N_2) temperature 200 ºC, drying gas flow rate 4 $L \cdot min^{-1}$, nebulizer gas (N_2) 15 psi, fragmentor voltage 190 V, skimmer voltage 70 V and OCT 1 RF Vpp voltage 300 V. Data were collected in profile (continuum) at 1 spectrum/s (approx. 10,000 transients/spectrum) between m/z 100 and 3200, at the highest resolution mode (4 GHz). MassHunter Workstation software (Agilent Technologies) was used for CapLC-MS control, data acquisition and analysis.

2.4 Intact glycoprotein analysis

2.4.1 Capillary electrophoresis (CE)

An HP ^{3D}CE instrument (Agilent Technologies, Wilmington, DE, USA) equipped with a DAD was used for CE analysis. Instrument control, data acquisition and data processing were performed using ChemStation software (Agilent Technologies). Bare fused-silica capillaries 50 µm id 360 µm od were purchased from Polymicro Technologies (Phoenix, AZ, USA). The detection window was placed at 8.5 cm from the outlet of the capillary. Experimental conditions for the analysis of rhEPO following the Eur.Ph. recommendations have been described elsewhere [24–26]. In our case, separations were performed using a capillary with a total length of 68.5 cm. The temperature was set at 35ºC. Samples were injected hydrodynamically at 33.5 mbar for 15 sec. A voltage of 15 kV (normal polarity) was applied during electrophoretic separations and detection was performed at 214 nm. The background electrolyte (BGE) for the high sialic acid content rhEPOs contained: 0.01 M tricine, 0.01 M NaCl, 0.01 M NaAc, 7 M urea, 2.5 mM putrescine and adjusted to pH 5.5 with 2M acetic acid (AC

BGE). The BGE optimized for NeuroEPO plus contained: 0.01 M malonic acid, 0.01 M NaCl, 7 M urea, 2.5 mM putrescine and adjusted to pH 6.0 with 1M NaOH (MAL BGE). All capillary rinses were performed at 930 mbar. New capillaries were activated by flushing with 1 M NaOH (20 min), water (20 min) and BGE (20 min). Between workdays or changes in BGE composition, capillaries were rinsed with water (5 min) and BGE (10 min) and 15 kV was applied for 10 min. Between runs capillaries were rinsed with water (5 min) and BGE (10 min). Putrescine-coated capillaries were stored overnight filled with water. If a longer storage was necessary, the capillary was rinsed with water for about 10 min and dried by flushing air for 10 min. Solutions were passed through a 0.45 µm nylon filter (MSI, Westboro, MA, USA).

2.4.2 IEF-PAGE

An automatic equipment for horizontal electrophoresis, "PhastSystem" (GE Pharmacia Biotech, EU) was used for IEF-PAGE analysis. Dehydrated polyacrylamide gels (Phast Gel™ Dry IEF, Cytiva, Sweden) were soaked in a solution mixture of Pharmalytes® IEF carrier ampholytes (86:14 (v/v) pH 2.5-5:3-10) and 7M urea. For all the evaluated samples, 4 µg of rhEPO was applied per lane and a low range pI (pH 2.5-6.5) calibration kit was used for pI determination (Amersham, GE Healthcare, UK). The running conditions in the "PhastSystem" were 2.5 mA, 750 V at 15ºC. After IEF-PAGE, silver staining was performed. Densitometric analysis of gels were carried out with a Syngene 900 calibrated imaging densitometer and data processing was performed using GeneTools software version 4.3.8.0 (Syngene, Cambridge, UK).

3 Results and discussion

3.1 N-glycan analysis

Some recombinant erythropoietin products have been described in the literature to show excellent neuroprotective properties without erythropoietic activity due to their low sialic acid content [8, 13, 14]. However, no studies regarding the glycan composition of these nonerythropoietic erythropoietins have been performed up to now. In this work, a bottom-up approach was used to characterize NeuroEPO plus glycosylation. Its N-glycan profile was compared to the one obtained for EPOCIM by CapZIC-HILIC-MS in combination with glycan isotope coded labeling through [$^{12}C_6$]/[$^{13}C_6$]-aniline for glycan relative quantitation [22]. EPOCIM is a commercially available erythropoietic rhEPO also produced by the CIM and it is widely used as biopharmaceutical. For this purpose, three independent samples (n=3) of NeuroEPO plus were labeled with [$^{12}C_6$]-aniline, while three samples of EPOCIM were labeled with [$^{13}C_6$]-aniline. After derivatization, equimolar mixtures of NeuroEPO plus and EPOCIM were prepared and analysed by CapZIC-HILIC-MS. Fig. 1 shows the results obtained for the most abundant bi- and tetraantennary glycans by way of an example. Nomenclature used for glycans corresponds to their composition in terms of number of hexoses (H), N-acetylglucosamines (N), fucoses (F) and sialic acids (S). The extracted ion chromatograms (EICs) of the glycans detected in NeuroEPO plus and EPOCIM are illustrated in red and blue colours, respectively. As can be observed, the most abundant tetraantennary glycan in NeuroEPO plus and EPOCIM was H7N6F1S3 and H7N6F1S4, respectively (Fig. 1A(i)), while NeuroEPO plus showed a superior amount of biantennary glycans compared to EPOCIM (Fig. 1B(i)). The presence of only one peak per glycan suggested the absence of isobaric isomers due to different sialic acid linkage-types, unlike what observed in endogenous glycoproteins [22, 27]. Moreover, as shown in the mass spectrum of each glycan (Fig. 1A(ii) and Fig. 1B(ii)), isotope

labeled species differ by 6 mass units, allowing quantifying without interference from the differentially labeled counterpart. Table 1 shows all the detected N-glycans in NeuroEPO plus and EPOCIM, and the experimental monoisotopic molecular masses (M_{exp}) with their mass errors for the labeled glycans detected in NeuroEPO plus. To properly compare the differences between the glycosylation profile of both glycoproteins, an average ratio of each glycan was determined in the three binary mixtures, considering the peak area of the glycan in NeuroEPO plus divided by the peak area of the same glycan in EPOCIM. The obtained values confirmed the superior proportion of hyposialylated glycans in NeuroEPO plus compared to EPOCIM (see Table 1). All mono- and di-sialylated glycans showed ratios above 1, being their amount higher than 10-fold for H6N5F1S1 and H7N6F1S1 glycans. Moreover, glycans without sialic acids, not previously described in other rhEPOs, were detected in NeuroEPO plus (H5N4F1 and H7N6F1) [28, 29]. Other authors compared the glycan mapping of several rhEPOs by the analysis of free or labeled glycans by CE-MS or LC-MS approaches [28, 29]. However, for a reliable and reproducible comparison between glycan profiles, we strongly recommend the use of a glycan isotope coded labeling strategy to avoid a misleading glycan quantitation, especially when we need to detect minor differences in the glycosylation pattern of two proteins.

Finally, relative areas (%A) were also calculated (peak area of each glycan divided by de sum of peak areas of all glycans detected in the studied glycoprotein) to have an estimation of the percentage of sialylation and type of branching of the studied glycoproteins (see Table 1), despite the limitations in quantitation when considering similar ionization yields between glycans with different sialic acid content. Around 40% of NeuroEPO plus glycans contained 1 or 2 sialic acids, whereas in EPOCIM these glycans only accounted for 10% (~90 % of EPOCIM detected glycans were tri- and tetrasialylated). Regarding branching, although similar abundance of tetraantennary glycans was detected in both glycoproteins (41.3% *versus* 44.8%), NeuroEPO plus

showed an increase in bi- and triantennary structures to detriment of tetraantennary with additional LacNAc units. This characteristic glycosylation detected in NeuroEPO plus could explain the higher efficiency of this nonerythropoietic erythropotein in the mechanisms of neuroprotection and neuroregeneration described *in vitro* and *in vivo* [17].

3.2 Intact glycoprotein analysis

Once differences in the levels of sialylation and glycan branching between NeuroEPO plus and conventional rhEPOs were confirmed, the CE-UV method proposed by the Eur.Ph. was evaluated for the analysis of NeuroEPO plus intact glycoforms [23, 24, 30, 31]. As in previous studies, a capillary column with a total length of 68.5 cm was used to reduce analysis time without detriment to glycoform separation [30, 31]. Fig. 2 shows the electropherograms corresponding to the Eur.Ph. reference standard (EPO-CRS), EPOCIM and NeuroEPO plus. Under these conditions, a baseline resolution of eight bands was obtained for EPO-CRS and EPOCIM (Fig. 2A and Fig. 2B). These rhEPO products show the same number of glycoform bands but different distribution. In the case of NeuroEPO plus, only two glycoform bands were detected (Fig. 2C). As NeuroEPO plus has lower sialic acid content, most of its glycoforms were neutral at pH 5.5, and consequently, less bands were observed as they migrated with the electroosmotic flow (EOF). The obtained results confirmed that the employed acetic acid/acetate background electrolyte (AC BGE) required an optimization for improving CE separation of such basic rhEPO product. First, the pH of the AC BGE was increased to promote glycoforms being negatively charged and migrating against the EOF. Fig. 3A(i-iii) show the electrophoretic separation of NeuroEPO plus at pH 6.0, 6.2 and 6.4, respectively. As can be observed, a superior number of bands were detected at pH 6.0-6.2 compared to 5.5. However, higher pH values provided poor resolution and distortion

of the glycoform bands, probably due to the low buffering capacity of the AC BGE at these pH values. Hence, other biological buffers were tested at several concentrations and pH values (e.g. Malonic acid, ACES and Bis-Tris, all of them with a pKa ~ 6). Malonic acid/malonate buffer (MAL BGE) gave the best results. The presence of tricine, urea and the rest of additives, described for improving rhEPO separation in the Eur.Ph. method [24, 26], was also evaluated in MAL BGE. To achieve an adequate separation, all these additives were required with the exception of tricine. Fig. 3B(i-iii) shows NeuroEPO plus separation using MAL BGE (0.01 M malonic acid, 0.01 M NaCl, 7 M urea, 2.5 mM putrescine) adjusted to pH 6.0, 6.2 and 6.4. At the same pH value (pH 6.0), the number of detected bands increased compared to the AC BGE recommended by the Eur.Ph. (see Fig. 3A(i) and 3B(i)). This fact suggests that not only the pH of the BGE, but also other factors such as the interaction of malonic acid/sodium malonate with the intact protein favoured glycoform separation. To improve resolution and detect additional bands of NeuroEPO plus, changes in the capillary length and separation voltage, as well as higher concentrations of malonic acid and putrescine were tested at several pH values. Nevertheless, the best results were those obtained with MAL BGE at pH 6.0 and it was selected for NeuroEPO plus separation. Although the MAL BGE at pH 6.0 was also tested, the AC BGE at pH 5.5 provided better resolution and glycoform separation for conventional rhEPO products, as can be observed for EPOCIM in Fig. S1. Table 2 shows the intraday reproducibility in terms of relative standard deviations (RSDs) of migration times (t_m) and relative areas (%) for each EPOCIM and NeuroEPO plus glycoform with the optimum method. As can be observed, the intraday reproducibility of both methods was comparable, taking the special precaution of refreshing the MAL BGE after three analyses. Reproducibility of migration times was excellent, and RSDs of relative areas were almost always lower than 5%, except for the first glycoform band of each glycoprotein due to their low abundance.

Finally, IEF-PAGE experiments were performed to establish a complementary method to CE-UV for the quality control of NeuroEPO plus and other hyposialylated erythropoietins. A pH gradient of 2-6 was commonly used for the separation of rhEPO products by IEF [23]. In this study, several proportions of ampholytes and pH conditions were tested. A solution mixture of ampholytes 86:14 (v/v) (pH 2.5-5:3-10), which provides a pH gradient of ~ 2.5-6.5, was selected to simultaneously separate NeuroEPO plus and conventional rhEPO products with higher sialic acid content (EPO-CRS or EPOCIM). Fig. 4A shows the IEF-PAGE glycoform separation obtained for EPOCIM and two different batches of NeuroEPO plus. A total of 8 and 6 main glycoform bands were separated in EPOCIM and NeuroEPO plus, respectively, in agreement with the results obtained by CE-UV. NeuroEPO plus, unlike the NeuroEPO previously reported by [13, 32], showed a lower amount of bands with a narrower range of pI values (from 4.25 to 5.85) due to the different fermentation and purification process of this new recombinant product. As can be observed in the gel of Figure 4A, NeuroEPO plus showed two concomitant bands with EPOCIM (bands 5 and 6 of NeuroEPO plus with bands 1 and 2 of EPOCIM). This overlapping profile was also observed when analysing both glycoproteins by the Eur.Ph. CE-UV method (see Fig. 2B and Fig. 2C). Furthermore, densitometric analysis of the gels confirmed that CE-UV and IEF-PAGE methods gave very similar NeuroEPO plus profiles (see Fig. 3B(i) and Fig. 4B). Hence, although IEF-PAGE offered lower reproducibility, as can be observed in the error bars of the relative areas (%) of Fig. 4C, the glycoform quantitation achieved was comparable, being able to use both developed methods in the quality control of this hyposialylated EPO.

4 Conclusions

In the first part of this paper, CapZIC-HILIC-MS in combination with a GRIL strategy with [$^{12}C_6$]/[$^{13}C_6$]-aniline was used, for the first time, to deeply characterize the N-glycans of a nonerythropoietic erythropoietin. The superior amount of glycans with lower sialic acid content and less branched structures detected in NeuroEPO plus could be key points to delve into the neuroprotective and neuroregenerative potential of this rhEPO product. In the second part, a new CE-UV method based on a malonic acid/sodium malonate buffer at pH 6.0 was developed for the separation of NeuroEPO plus intact glycoforms. Additionally, an IEF-PAGE method was established for the simultaneous analysis of NeuroEPO plus and conventional acidic rhEPO products. Both methods provide reference glycoform patterns of NeuroEPO plus to ensure manufacturing consistency and product safety of this novel product, but also the developed methods could be applied to other highly basic rhEPOs, where the European Pharmacopoeia CE methodology fails. The proposed glycomic and intact glycoprotein methods provide a robust and reliable characterization of NeuroEPO plus and, in the future, an analytical platform for its application as biopharmaceutical in neurodegenerative diseases.

Author Contributions

Designed the study (J.A.G-A, M.M.-A., E.G., T.R. and V.S.-N.), performed GRIL−mass spectrometry and data analysis (M.M.-A.), performed CE-UV and IEF-PAGE analysis (J.A.G-A and E.G.), wrote the manuscript (M.M.-A., J.A.G-A and E.G.). All authors reviewed the manuscript.

The authors declare no competing financial interest.

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Figure Captions

Fig.1. CapZIC-HILIC-MS analysis of a mixture (1:1) of aniline-labeled N-glycans released from 25 µg of EPOCIM (labelled with [$^{13}C_6$]-aniline) and 25 µg of NeuroEPO plus (labelled with [$^{12}C_6$]-aniline): (A) EICs of the main tetrantennary glycans; (B) EICs of the main biantennary glycans (EPOCIM, in blue, and NeuroEPO plus, in red) with the negative ion mass spectra over the time window corresponding to the indicated peaks (A(i) and B(i)).

Fig.2. CE separation of rhEPO glycoforms using the separation buffer recommended by the Eur.Ph. method (sample: 1000 ppm of filtered rhEPO; injection: 15 s, 33.5 mbar; voltage: 15 kV; *T*: 35ºC, λ: 214 nm; bare-fused silica capillary: 68.5 cm x 50 µm; separation buffer: 0.01 M Tricine, 0.01 M NaCl, 0.01 M NaAc, 7 M urea, 2.5 mM putrescine, pH 5.5 with 2 M HAc). (A) EPO-CRS, (B) EPOCIM, (C) NeuroEPO plus.

Fig.3. CE separation of NeuroEPO plus glycoforms at several pH values using (A) the separation buffer recommended by the Eur.Ph. method (AC BGE: 0.01 M Tricine, 0.01 M NaAc, 0.01 M NaCl, 7 M urea, 2.5 mM putrescine, adjusted with 2 M HAc) and (B) a separation buffer of malonic acid/malonate (MAL BGE: 0.01 M malonic acid, 0.01 M NaCl, 7 M urea, 2.5 mM putrescine, adjusted with 1 M NaOH). (i) pH 6.0, (ii) pH 6.2, (iii) pH 6.4.

Fig.4. (A) IEF-PAGE separation of EPOCIM and NeuroEPO plus glycoform bands (B) NeuroEPO plus IEF glycoform profile obtained by densitometry (C) Comparison of glycoform band quantitation of NeuroEPO plus obtained by CE-UV and IEF-PAGE developed methods. In both cases, the relative area (%) of each glycoform band was calculated normalizing against the sum of the peak areas of all glycoform bands. Standard deviations associated for both methods after three replicate analysis of the same sample (n=3) are also depicted in the graph.

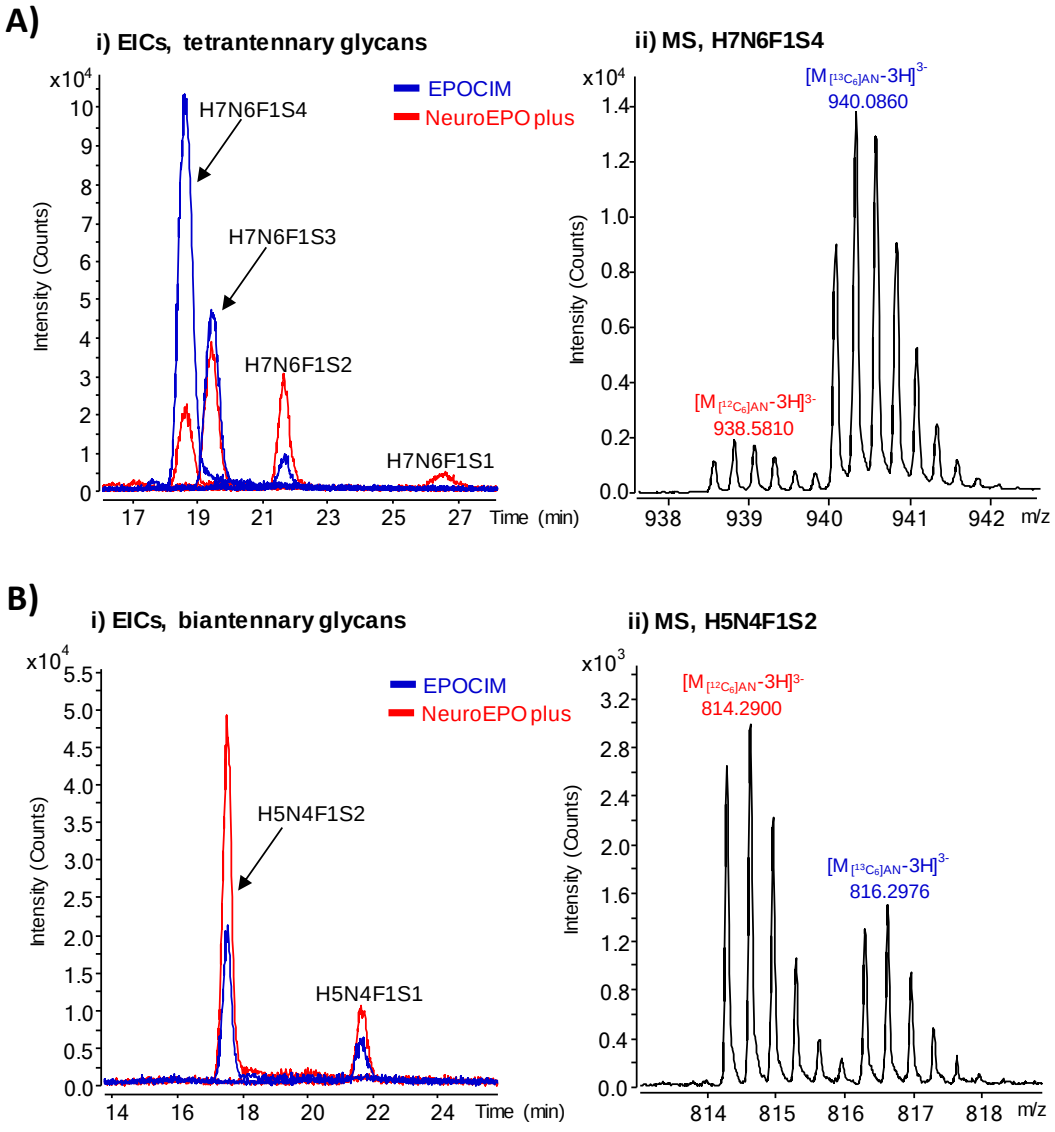

A)
i) EICs, tetrantennary glycans
ii) MS, H7N6F1S4
EPOCIM
NeuroEPO plus
H7N6F1S4
H7N6F1S3
H7N6F1S2
H7N6F1S1
Intensity (Counts)
Time (min)
[M [13C6]AN-3H]3-
940.0860
[M [12C6]AN-3H]3-
938.5810
m/z
B)
i) EICs, biantennary glycans
ii) MS, H5N4F1S2
EPOCIM
NeuroEPO plus
H5N4F1S2
H5N4F1S1
[M [12C6]AN-3H]3-
814.2900
[M [13C6]AN-3H]3-
816.2976

Figure 1

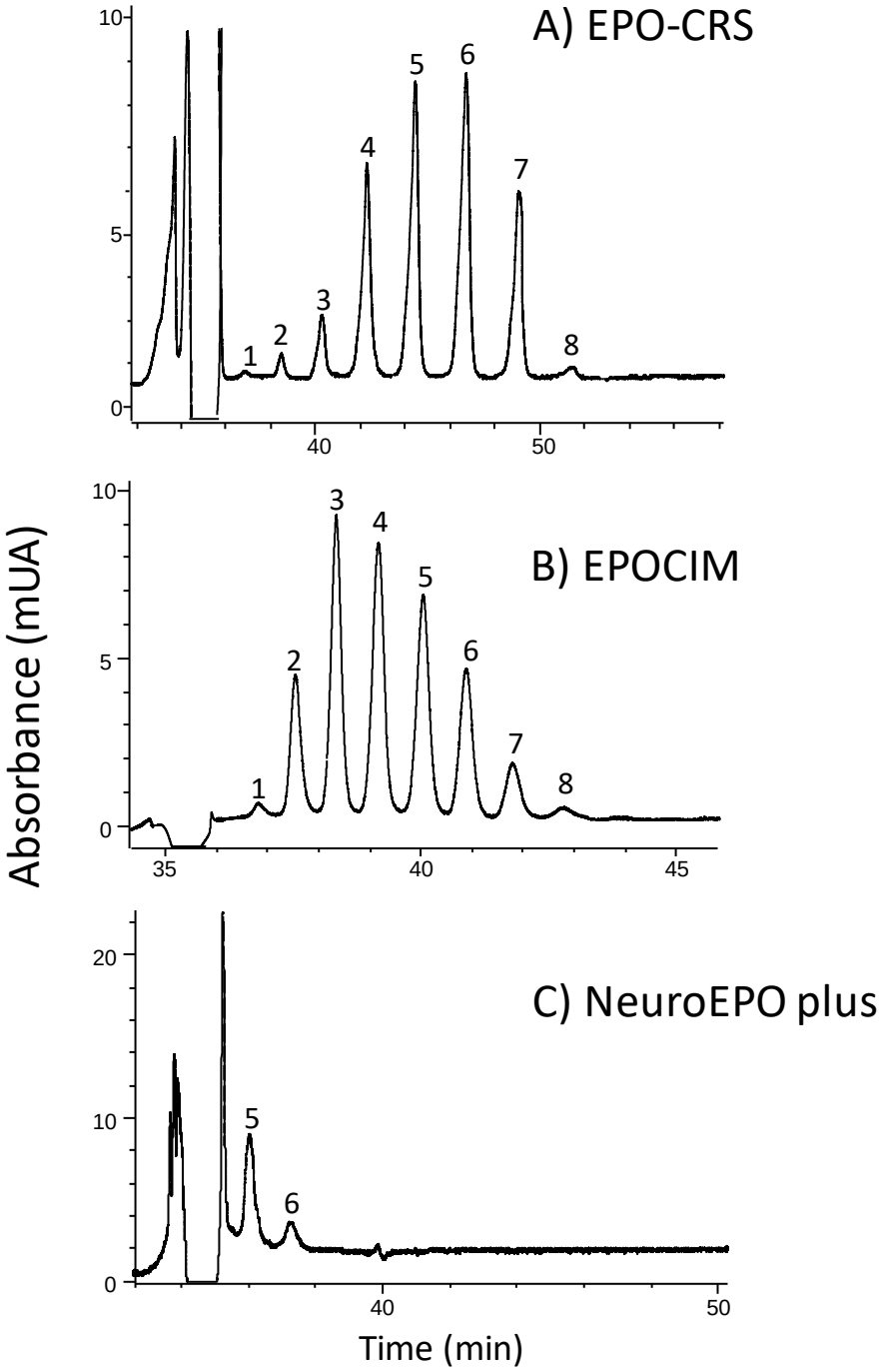

A) EPO-CRS
B) EPOCIM
C) NeuroEPO plus
Absorbance (mUA)
Time (min)
1
2
3
4
5
6
7
8

Figure 2

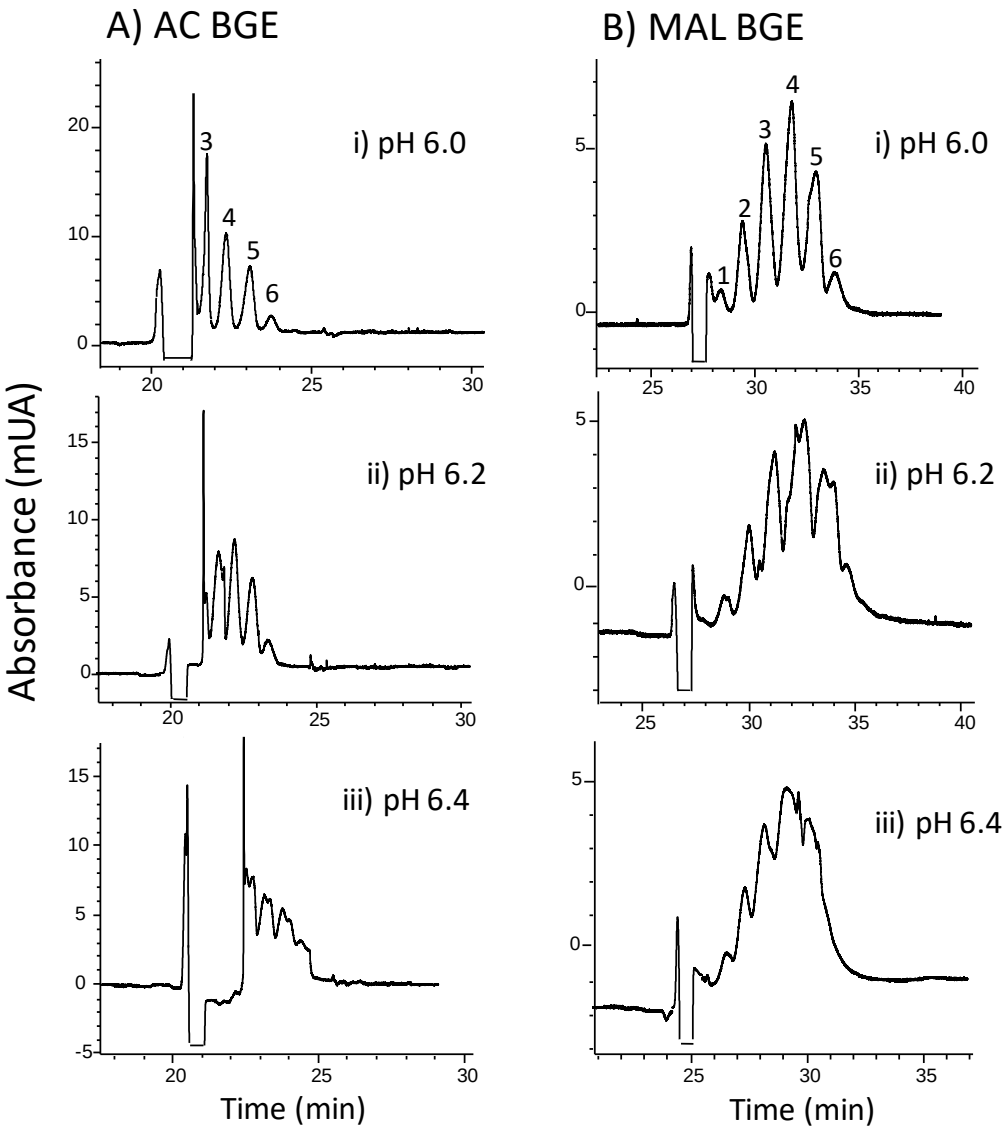
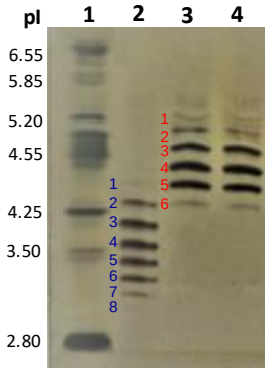

A) AC BGE
B) MAL BGE
i) pH 6.0
ii) pH 6.2
iii) pH 6.4
Absorbance (mUA)
Time (min)
Time (min)

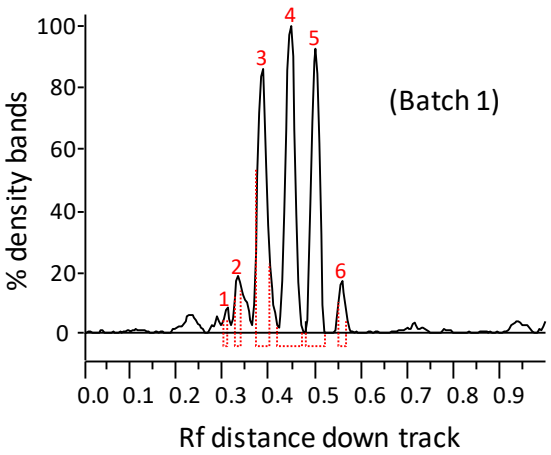
Figure 3

A) IEF-PAGE



1. pI marker
2. EPOCIM
3. NeuroEPO plus (batch 1)
4. NeuroEPO plus (batch 2)

B) NeuroEPO plus glycoform profile



C) NeuroEPO plus glycoform quantitation

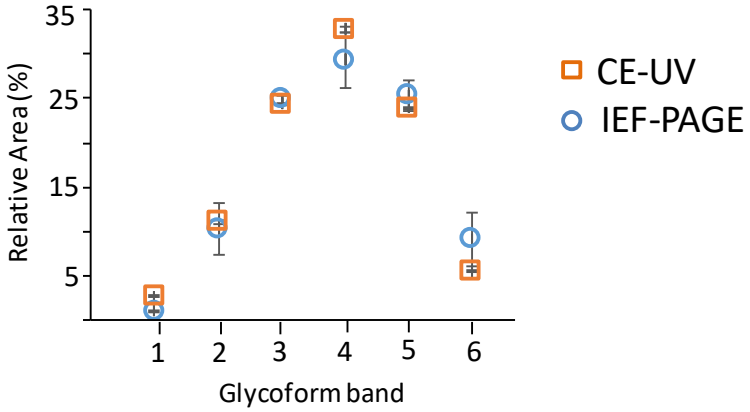


Figure 4

Table 1: *N*-glycans detected by CapZIC-HILIC-MS from equimolar mixtures of NeuroEPO plus glycans labeled with [$^{12}C_6$]AN and EPOCIM glycans labeled with [$^{13}C_6$]AN (25µg of each protein). Glycan ratios and relative areas (%A) obtained from three independent binary mixtures (n=3).

Glycan [a]	M_{exp} (Da) [b]	Error (ppm)	Glycan ratio [c]		Relative Area (%A) [d]			
					NeuroEPO plus		EPOCIM	
			Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
H5N4F1	1863.6892	14.0	-	-	0.4	4.6	0.0	-
H5N4F1S1	2154.7846	6.4	1.6	4.8	2.8	8.8	1.3	4.5
H5N4F1S2	2445.8800	7.3	2.4	0.8	11.6	1.5	3.7	2.6
Total 2Ant					**14.8**	0.8	**4.9**	1.6
H6N5F1S1	2519.9168	4.0	10.6	4.5	1.8	3.6	0.1	8.4
H6N5F1S2	2811.0122	6.1	2.6	5.9	7.2	0.4	2.1	6.0
H6N5F1S3	3102.1076	5.1	1.2	1.8	12.1	1.8	7.3	2.7
Total 3Ant					**21.0**	1.6	**9.5**	3.2
H7N6F1	2593.9536	-2.9	-	-	0.1	12.2	0.0	-
H7N6F1S1	2885.0490	6.7	11.6	6.4	2.1	5.7	0.1	16.9
H7N6F1S2	3176.1444	3.5	3.1	2.8	9.2	1.8	2.2	2.6
H7N6F1S3	3467.2398	4.6	1.2	2.1	22.2	1.1	13.3	3.0
H7N6F1S4	3758.3353	5.3	0.2	1.6	7.9	1.0	29.1	2.6
Total 4Ant					**41.3**	0.4	**44.8**	1.5
H8N7F1S1	3250.1812	4.2	-	-	1.0	20.3	0.0	-
H8N7F1S2	3541.2766	2.9	2.8	4.8	3.0	1.8	0.8	3.3
H8N7F1S3	3832.3720	5.2	0.9	3.8	9.2	4.1	8.0	1.5
H8N7F1S4	4123.4675	6.9	0.2	6.3	4.4	6.0	19.9	2.0
Total 4Ant1LacNAc					**17.6**	2.7	**28.7**	1.7
H9N8F1S2	3906.4088	2.5	3.1	6.8	0.8	13.2	0.2	4.9
H9N8F1S3	4197.5042	0.5	0.9	3.5	2.3	4.4	2.0	3.0
H9N8F1S4	4488.5996	1.7	0.1	3.0	1.3	3.8	8.7	0.5
Total 4Ant2LacNAc					**4.4**	5.3	**10.9**	0.9
H10N9F1S3	4562.6364	4.2	1.0	4.1	0.3	3.6	0.3	7.2
H10N9F1S4	4853.7318	-13.8	0.2	5.3	0.3	5.6	0.9	0.3
Total 4Ant3LacNAc					**0.6**	4.3	**1.2**	1.8

[a] Nomenclature used for glycans correspond to their composition in terms of number of hexoses (H), N-acetylglucosamines (N), fucoses (F) and sialic acids (S).

[b] M_{exp}: Experimental monoisotopic molecular mass for the glycan labeled with [$^{12}C_6$]AN (NeuroEPO plus sample).

[c] Glycan ratios obtained from 3 independent binary mixtures of [$^{12}C_6$]AN-NeuroEPO plus glycans and [$^{13}C_6$]AN-EPOCIM glycans (n = 3). Each glycan ratio was calculated as: EIC peak area glycan-[$^{12}C_6$]AN divided by EIC peak area glycan-[$^{13}C_6$]AN. A ratio value of 1 is obtained when the glycan in NeuroEPO plus had the same peak area as the glycan detected in EPOCIM. Ratios of glycans that were not detected in EPOCIM could not be calculated (i.e. H5N4F1, H7N6F1 and H8N7F1S1).

[d] Relative area (%A) obtained from 3 independent binary mixtures of [$^{12}C_6$]AN-NeuroEPO plus glycans and [$^{13}C_6$]AN-EPOCIM glycans (n = 3): Each relative area (%A) was calculated as: EIC peak area of each glycan divided by de sum of EIC peak areas of all glycans detected in the studied glycoprotein

Table 2. Migration times and relative areas with their corresponding relative standard deviations (RSDs) of EPOCIM glycoforms by the Eur.Ph. CE-UV method (AC BGE, pH 5.5) and NeuroEPO plus glycoforms by the established CE-UV method (MAL BGE, pH 6.0).

EPOCIM glycoforms (AC BGE, pH 5.5)	**t_m (min)**	**RSD (%)**	**Relative area* (%A)**	**RSD (%)**
1	36.7	0.2	0.9	13.9
2	37.3	0.1	10.3	2.4
3	38.1	0.1	23.3	1.2
4	38.9	0.1	24.1	1.3
5	39.8	0.1	20.4	0.7
6	40.7	0.1	14.0	1.6
7	41.6	0.1	5.7	2.2
8	42.6	0.1	1.4	5.5
NeuroEPO plus glycoforms (MAL BGE, pH 6.0)	**t_m (min)**	**RSD (%)**	**Relative area* (%A)**	**RSD (%)**
1	29.0	0.7	2.7	11.3
2	30.5	0.6	11.1	4.2
3	32.2	0.5	24.3	1.7
4	34.1	0.5	32.9	2.2
5	35.9	0.6	23.9	0.9
6	37.4	0.6	5.5	3.6

* Each relative area was calculated as the peak area of each glycoform divided by de sum of peak areas of all glycoforms detected in the studied glycoprotein. Six replicate analysis of the same sample in the same day (n=6).

Supporting Information

for

CHARACTERIZING A NOVEL HYPOSIALYLATED ERYTHROPOIETIN BY GLYCAN AND INTACT GLYCOPROTEIN ANALYSIS

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Fig.S1. Comparison of EPOCIM glycoform band separation by CE-UV using (a) AC BGE at pH 5.5 and (EurPh. method) and (b) MAL BGE at pH 6.0 (method established for NeuroEPO plus).