Received: 2 March 2011

Revised: 15 April 2011

Accepted: 22 May 2011

Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. 2011, 25, 2307–2316 (wileyonlinelibrary.com) DOI: 10.1002/rcm.5114

Capillary electrophoresis time-of-flight mass spectrometry for a confident elucidation of a glycopeptide map of recombinant human erythropoietin

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Capillary electrophoresis coupled to orthogonal accelerated time-of-flight mass spectrometry (CE/TOFMS) was used for the analysis of *O*- and *N*-glycopeptides of recombinant human erythropoietin (rhEPO). O₁₂₆ and N₈₃ with a tetraantennary complex type glycan (N₈₃-4Ant) were selected as glycopeptide models to develop an optimum CE/ TOFMS methodology capable of detecting and characterizing the wide variety of glycopeptides present in the glycoprotein digest. Glycopeptide adsorption in the inner surface of the fused-silica capillary was prevented after using a capillary conditioning of 1 M HAc between runs. On the other hand, different acidic conditions in the sheath liquid (SL) and in the background electrolyte (BGE) were tested with the aim of studying their influence in glycopeptide fragmentation. Finally, the fragmentor voltage value of the TOF-MS instrument was optimized to avoid the involuntary fragmentation of the native glycopeptides. Hence, the established method may be regarded as an excellent starting point to obtain reliable glycopeptide maps of complex glycoproteins such as rhEPO by CE/TOFMS. Copyright © 2011 John Wiley & Sons, Ltd.

Glycosylation is one of the most common post-translational modifications in proteins. It has been estimated that at least 50% of the proteins in mammalian proteomes are glycosylated. Glycoproteins consist of mixtures of glycosylated variants, known as glycoforms, which share the same peptide sequence but show different oligosaccharides at their characteristic O- or N-glycosylation sites. Glycoproteins play a crucial role in recognition, signaling and adhesion processes on the cell surfaces. Many of these functions are mediated by the oligosaccharide chains. Moreover, O- and N-glycan chain structures of glycoproteins may be altered in many diseases such as congenital disorders of glycosylation, alcoholism or cancer.^[1] Hence, detection and characterization of the different oligosaccharides attached to a given glycoprotein invokes great interest in many research fields such as biotechnology, biochemistry, medicine and pharmacology. The analysis of a glycoprotein carbohydrate microheterogeneity is usually carried out at the glycan or glycopeptide level after chemical or enzymatic digestion. In contrast to glycans, glycopeptides not only provide information about the structure and composition of the oligossacharides, but also allow information to be obtained about glycosylation sites and their degree of occupation.

In recent years, capillary electrophoresis coupled to mass spectrometry (CE/MS) has been used in glycoproteomics for the analysis of intact glycoproteins, as well as for glycopeptides and glycans from protein digests.^[2-10] CE offers the possibility of separating these biomolecules according to their mass-to-charge ratios (m/z) while MS detection enables elucidation of their molecular masses and structures. Orthogonal acceleration time-of-flight (oaTOF) mass spectrometers are excellent MS analyzers for the study of glycopeptides, especially those of high molecular mass, as they provide accurate and high-resolution mass measurements over wide mass-to-charge acquisition ranges permitting the detection of the multiply charged molecular ions expected for this kind of complex analyte. However, the analysis of glycopeptides by CE/MS is a challenging task. On the one hand, they are difficult to ionize in positive ESI mode, especially if they contain glycans with negatively charged sialic acid residues, or ion suppression occurs because the separation from the other peptides and glycopeptides in the digest is not complete. On the other hand, glycopeptides may undergo fragmentation of labile groups such as sialic acids (SiA), hexoses (Hex), deoxysugars such as fucose or hexose-N-acetylhexose units (HexHexNAc) depending on the MS detection conditions. This involuntary fragmentation is in general overlooked and may mislead glycoproteomics researchers, leading to wrong conclusions about glycopeptide composition of a certain glycoprotein. For these reasons, new CE/MS methodology needs to be established in order to improve glycopeptide analysis in terms of separation and MS detection. The goal may be separation and detection of the maximum number of

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glycopeptides of a complex protein digest, while preventing their fragmentation to obtain a reliable map for a confident glycoprotein characterization.

In this work, with the aim of developing an optimal methodology for the analysis of glycopeptides in enzymatic protein digests by CE/TOFMS, recombinant human erythropoietin (rhEPO) has been selected as a model. rhEPO is a biologically and therapeutically relevant glycoprotein. Approximately 40% of its molecular mass (29 888 Da, the most abundant glycoform^[2]) is composed of carbohydrates, of which 17% correspond to sialic acids, mainly N-acetylneuraminic acid (Neu5Ac). The wide variety of glycans are attached to three N-glycosylation sites at Asn 24, 38 and 83 and one O-glycosylation site at Ser 126.^[11] The complex peptide and glycopeptide mixture obtained after enzymatic digestion is similar to those that can be expected for other complex glycoproteins. Moreover, the analysis of rhEPO glycopeptides gives rise to great interest in doping analysis and quality control to find, in the future, discrimination points between the endogenous and the recombinant erythropoietins. The study is focused on the O₁₂₆ glycopeptide and also N₈₃ with a tetraantennary complex type glycan (N₈₃-4Ant) glycopeptide. Both glycopeptides show a great number of structural differences and may be useful to develop a general method for detection and characterization of the wide variety of glycopeptides present in a glycoprotein digest.

EXPERIMENTAL

Chemicals

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Isopropanol (iPrOH), acetic acid (HAc, glacial), formic acid (HFor, 98–100%), ammonia (25%), ammonium acetate (NH₄Ac) and sodium hydroxide were supplied by Merck (Darmstadt, Germany). DL-Dithiothreitol (DTT, \geq 99%), iodoacetamide (IAA) and ammonium hydrogen carbonate (NH₄HCO₃) were supplied by Sigma-Aldrich (Madrid, Spain). Trypsin (sequencing grade modified) from Promega (Madison, WI, USA) was obtained from Roche (Mannheim, Germany). Water with a conductivity lower than 0.05 mS/cm was obtained using a Milli-Q water purification system from Millipore (Molsheim, France).

ESI Low Concentration (ESI-L) tuning mix was supplied by Agilent Technologies (Waldbronn, Germany) for tuning and calibration of the oaTOF mass spectrometer. The reference compound solutions for internal mass recalibration of the TOF mass spectrometer (2.5 mM HP-0921, hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine; 0.2 mM HP-1221, hexakis(1*H*,1*H*,4*H*hexafluorobutyloxy)phosphazine; 0.2 mM HP-1821, hexakis (1*H*,1*H*,6*H*-decafluorohexyloxy)phosphazine; 0.5 mM HP-2421, hexakis(1*H*,1*H*,8*H*-tetradecafluoroctyoxy)phosphazine in acetonitrile) were also supplied by Agilent Technologies.

rhEPO samples

rhEPO produced in a Chinese hamster ovary (CHO) cell line was provided by the European Pharmacopoeia as a Biological Reference Product (BRP-lot2 and lot3). Each sample vial contained 250 µg of EPO (a mixture of epoetin alpha and beta), 0.1 mg of Tween 20, 30 mg of trehalose, 3 mg of arginine,

4.5 mg NaCl, and 3.5 mg of Na₂HPO₄. The content of each vial was dissolved in purified water to obtain a 1000 mg·L⁻¹ solution of rhEPO. Excipients of low molecular mass were removed from the rhEPO sample by passage through a Microcon YM-10 centrifugal filter from Millipore (Mr cut-off 10 kDa, Bedford, MA, USA).^[10] The filter was initially washed with purified water for 10 min in a centrifuge at 13 000 g, and the sample was centrifuged after that for 10 min under the same centrifugal force. The residue was washed three times for 10 min in the same way with an appropriate volume of purified water. The final residue was recovered from the upper reservoir by upside-down centrifugation in a new vial (3 min at 1000 g) and sufficient purified water was added to adjust the rhEPO concentration to 1000 mg·L⁻¹.

rhEPO was reduced, alkylated and immediately subjected to enzymatic digestion. Briefly, 2.5 µL of 0.5 M DTT in 50 mM NH₄HCO₃ (pH 7.9) were added to an aliquot of $100 \,\mu\text{L}$ of the desalted $1000 \,\text{mg} \cdot \text{L}^{-1}$ rhEPO solution. The mixture was incubated in a water bath at 56 °C for 30 min and then alkylated with 50 mM IAA for 30 min at room temperature in the dark (7 µL of 0.73 M IAA). Excess of low molecular weight reagents was removed with Microcon YM-10 centrifugal filters as explained before. In this case, the final residue was reconstituted in 100 µL of 50 mM NH₄HCO₃ (pH 7.9). For trypsin digestion, an enzyme-to-protein ratio of 1:40 by mass was added, and the mixture was carefully vortexed and incubated at 37 °C in a water bath for 18 h.^[10] Digestion was stopped by heating for 5 min in boiling water and stored at -20 °C until its use. rhEPO digest was analyzed by CE/ TOFMS injecting ~16 ng of digest for a single analysis. Digestion reproducibility was evaluated preparing two rhEPO digests from two different EPO BRP batches (lot2 and lot3). No differences could be observed between digests and lots by CE/TOFMS demonstrating the reproducibility of the enzymatic digestion procedure.

CE/TOFMS

The CE/TOFMS experiments were performed in the HP^{3D}CE system coupled to a 6220 oaTOF LC/MS mass spectrometer with an orthogonal G1603A sheath-flow interface (Agilent Technologies, Waldbronn, Germany).^[12] The sheath liquid was delivered at a flow rate of 3.3 µL·min⁻¹ by a KD Scientific 100 series infusion pump. CE control and separation data acquisition (e.g. voltage, temperature and current) were performed using ChemStation software (Agilent Technologies) that was running in combination with the MassHunter workstation software (Agilent Technologies) for control, data acquisition and processing of the oaTOF mass spectrometer. The oaTOF mass spectrometer was tuned and calibrated following the manufacturer's instructions. Once or twice per day, a 'Quick Tune' of the instrument was carried out in positive mode followed by a mass-axis calibration to ensure accurate mass assignments. Measurement parameters were tuned for the analysis of O₁₂₆ and N₈₃-4Ant glycopeptides paying special attention to the fragmentor voltage value. The optimum parameters were as follow: capillary voltage 4000 V, drying gas (N₂) temperature 200 °C, drying gas flow rate 4 L min⁻¹, nebulizer gas (N₂) 7 psig, fragmentor voltage 190 V, skimmer voltage 60 V, 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 working in the highest resolution mode (4GHz). The 'Molecular Feature Extractor' algorithm provided with the MassHunter workstation software was used for the identification of peptides and O_{126} and N_{83} -4Ant glycopeptides from rhEPO digests.

A Polymicro bare fused-silica capillary of 70 cm total length $(L_T) \times 50 \,\mu\text{m}$ internal diameter (i.d.) x 360 μm outer diameter (o.d.) supplied by Composite Metals Service (Worcester, UK) was used for CE/TOF-MS. New capillaries were activated by flushing them sequentially with the following solutions for 30 min each: 1 M NaOH, water and background electrolyte (BGE). Each capillary was conditioned every day by rinsing for 5 min with NaOH, 7 min with water and for 10 min with BGE. Activation and conditioning procedures were performed off-line in order to avoid NaOH entering the mass spectrometer. Electrophoretic separations were carried out at 25 °C under normal polarity (18 kV). Between runs two types of capillary conditioning were tested: A rinse of BGE for 5 min or rinsing the capillary for 1 min with water, 3 min with 1 M HAc, 1 min with water and 5 min with BGE. Columns were stored overnight filled with water.

Sheath liquids (SLs) of iPrOH/H₂O (50:50 v/v) with different amounts of HFor were tested: 0.01, 0.05 and 0.1% (v/v) of HFor. When internal mass recalibration was used, a SL containing HP-0921, HP-1221, HP-1821 and HP-2421 was prepared. 0.1, 1.4, 5.4 and 18 µL of each reference compound solution, respectively, were added to 25 mL of SL solution. All signal intensities of the reference compounds were around 5000–15 000 counts as recommended by the manufacturer.^[13] The sheath liquids were degassed for 10 min by sonication before use. For analysis of rhEPO digests, injection was performed hydrodynamically at 50 mbar for 15 s and two BGEs were used: an acidic BGE of 50 mM HAc and 50 mM HFor (pH 2.2) and a basic BGE of 50 mM NH₄Ac (pH 8.0). Before CE/MS, all solutions were passed through a 0.45-mm nylon filter (MSI, Westboro, MA, USA). All samples were kept at $4 \,^{\circ}$ C and stored at $-20 \,^{\circ}$ C when not in use for a long period.

pH measurements were performed with a Crison 2002 potentiometer and a Crison electrode 52–03 (Crison Instruments, Barcelona, Spain). Centrifugation procedures were carried out in a Mikro 20 centrifuge (Hettich, Tuttlingen, Germany).

RESULTS AND DISCUSSION

Analysis of rhEPO peptides

In a previous study, peptides obtained by trypsin-peptide: Nglycosidase F (PNGase) enzymatic digestion of rhEPO and NESP (novel erythropoiesis stimulating protein) glycoproteins were analyzed by capillary electrophoresis ion trap mass spectrometry (CE/ITMS) using an acidic background electrolyte (BGE) of 50 mM HAc and 50 mM HFor (pH 2.2).^[10] As oaTOF-MS provides higher sensitivity, mass accuracy and resolving power than ITMS,^[12] we analyzed rhEPO digested with trypsin (rhEPO (T)) by CE/TOFMS with the aim of increasing the coverage of the protein sequence obtained by CE/ITMS and also studying the different *O*- and *N*-glycopeptides contained in the rhEPO (T) sample (see next section). Figure 1 shows the extracted ion electropherograms (EIEs) for the detected peptides of rhEPO



(T) and Table 1 lists their corresponding amino acid position in the native protein sequence, their theoretical and experimental mass (M_{theo} and M_{exp}, respectively). As expected, separation did not significantly change from that obtained by CE/ITMS.^[10] Nevertheless, owing to the higher sensitivity of this mass spectrometer, peptides 13 and 14 were detected by CE/TOFMS increasing the coverage of the protein. Table 1 also shows the signal-to-noise ratio (S/N), as well as the relative standard deviations and average errors of the M_{exp} (RSD M_{exp} and Av. error, respectively) for each detected peptide with and without reference compounds in the SL. More accurate and reproducible Mexp values were obtained using references in the SL since the av. error and RSD M_{exp} values were lower in most of the cases. However, S/N values significantly decreased in the presence of references causing a total loss of signal for peptide 16. These results contrast with those we obtained for the analysis of neuropeptides in which S/N values were not significantly different with and without references in the SL.^[12] Hence, depending on the complexity of the sample to be analyzed, the use of references in the SL can be inconvenient for the detection of the analytes of interest by CE/TOFMS.

Analysis of O₁₂₆ and N₈₃-4Ant glycopeptides

In addition to peptides, enzymatic digestion of glycoproteins results in a heterogeneous mixture of glycopeptides which, in general, implies increased difficulties related to separation, detection and characterization. rhEPO (T) was analyzed by CE/TOFMS detecting different glycoforms of N₈₃, O₁₂₆ and N₂₄-N₃₈ glycopeptides (data not shown). Glycopeptides O₁₂₆ and N₈₃ with a tetraantennary complex type glycan $(N_{83}-4Ant)$ were selected as glycopeptide models to develop an optimum CE/TOFMS method capable of detecting and characterizing, in the future, the wide variety of glycopeptides present in the different recombinant and endogenous erythropoietins. rhEPO (T) permitted the detection of various glycopeptide glycoforms with different number of Neu5Ac for both O₁₂₆ and N₈₃-4Ant glycopeptides. Figure 2 shows the sum of EIEs of the main detected glycoforms for O₁₂₆ and N₈₃-4Ant glycopeptides with their corresponding mass spectra (Figs. 2(a) and 2(b), respectively). Three glycoforms of O₁₂₆ glycopeptide showing from 0 to 2 Neu5Ac were detected (Fig. 2(a), (ii, iii, iv)), whereas N₈₃-4Ant showed glycoforms from 2 to 4 Neu5Ac (Fig. 2(b), (ii, iii, iv)). In addition to these main glycoforms, acetylation of the Neu5Ac was also observed in the O₁₂₆ glycopeptide. O₁₂₆/1NeuAc showed non- and mono-acetylated glycoforms (see Fig. 2(a), (iii)) but we were not able to confirm the di-acetylated glycoform described by other authors, probably because its signal was too low to be differentiated from the background.[14-16] Moreover, O126/2Neu5Ac showed non- and mono-acetylated glycoforms (see Fig. 2(a), (iv)) but also di- and tri-acetylated glycoforms at a higher m/z range (data not shown). This contrasts with that described by Stübiger et al.[14] and Groleau et al.^[15] that only detected up to two acetylations of this glycopeptide form. On the other hand, glycoforms showing N-glycolylneuraminic acid (Neu5Gc) instead of Neu5Ac were also observed in O₁₂₆ but in very low abundance (e.g. Fig. 2 (a), (iv)). N₈₃-4Ant mass spectra showed higher complexity with great number of Na, K and NH₄ adducts due to the higher degree of glycosylation of this glycopeptide in



Figure 1. Extracted ion electropherograms (EIEs) of peptides from rhEPO (T) by CE/TOFMS. Peptide peak numbers are referred to their identification numbers presented in Table 1. (Sample: $1000 \text{ mg} \cdot \text{L}^{-1}$ of digested rhEPO; injection: 15 s at 50 mbar; voltage: +18 kV; T: 25 °C BGE: 50 mM HAc and 50 mM HFor pH 2.2. Fragmentor voltage 270 V).

Table 1. Peptides detected from rhEPO (T) by CE-TOFMS with and without reference compounds in the SL										
			WITH REFERENCE $(n=6)$			WITHOUT REFERENCE (n=6)				
Sequence ^a	Position	M _{Theo} (Da) ^b	S/N	M _{exp} (Da) ^b	RSD M _{exp} (%)	Av. error (ppm) ^d	S/N	M _{exp} (Da) ^b	RSD M _{exp} (%)	Av. error (ppm) ^d
1. APPR 2. LICDSR-	1-4 5-10	439,2543 762,3694	1732,5 1311,0	439,2540 762,3684	4,32 x10 ⁻⁵ 7,35 x10 ⁻⁵	0,68 1,29	3891,3 2768,0	439,2555 762,3732	3,27 x10 ⁻⁴ 8,89 x10 ⁻⁵	2,69 4,92
3. VLER 4. YLLEAK 5. VNFYAWK	11-14 15-20 46-52 54 76	515,3067 735,4167 926,4650 2525 2311	4537,3 8953,3 2200,0 3162.0	515,3065 735,4166 926,4644 2525 3190	4,82 x10 ⁻⁵ 1,70 x10 ⁻⁴ 1,23 x10 ⁻⁴ 4.96 x10 ⁻⁵	0,42 0,15 0,61	8136,4 14086,1 3190,2 4686 7	515,3072 735,4190 926,4691 2525 3110	$2,84 \times 10^{-4}$ $2,53 \times 10^{-4}$ $3,12 \times 10^{-4}$ 1.08×10^{-4}	1,03 3,08 4,38 7.95
0. MEVGQQA- VEVWQ GLALLSEAVLR 7 AVSCLR	98-103	601 3547	5844 7	601 3546	$4,90 \times 10^{-5}$	4,70	9349 3	601 3548	2.86×10^{-4}	0.17
8. SLTTLLR 9. ALGAQK	104-110 111-116	802,4912 586,3438	4537,4 4710,1	802,4919 586,3437	$5,75 \times 10^{-5}$ $5,15 \times 10^{-5}$ $3,24 \times 10^{-5}$ $5,68 \times 10^{-5}$	0,19 0,83 0,17	10809,2 6482,7	802,4946 586,3441	$7,55 \times 10^{-5}$ $3,46 \times 10^{-4}$	4,20 0,54 2,17
10. THADTPK 11. LFR 12. VYSNFLR	132-139 141-143 144-150	434,2641 897,4708	4116,7 3623,8	434,2640 897,4695	$4,70 \times 10^{-5}$ $7,34 \times 10^{-5}$	0,00 0,27 1,47	6095,0 4583,5	923,4732 434,2649 897,4749	$2,30 \times 10^{-4}$ $2,72 \times 10^{-4}$ $8,19 \times 10^{-5}$	2,17 1,84 4,57
13. GK 14. LK 15. LYTGEACR-	151-152 153-154 155-162	203,1270 259,1896 968,4385	646,1 1210,2 1615,3	203,1270 259,1896 968,4367	1,38 x10 ⁻⁵ 3,79 x10 ⁻⁵ 1,43 x10 ⁻⁵	0,17 0,06 1,86	326,8 1250,4 2916,8	203,1279 259,1898 968,4400	2,03 x10 1,45 x10 ⁻⁴ 2,94 x10 ⁻⁴	4,55 0,93 1,51
(Cys-CAM) ^e 16. TGDR	163-166	447,2077	-	-	-	-	59,9	447,1866	2,85 x 10 ⁻⁴	47,20
^a AA sequence of ^b M _{Theo} and M _{exp} ^c Cysteine was tree	EPO from monoisot eated with	n SwissProt. opic molecu iodoacetarr	llar mass lide to fo	es. rm carbamide	omethyl-cy	steine (C	vs-CAM)).		

^dAverage error was calculated as: $|(M_{exp}-M_{theo}) / M_{theo}|$

comparison with the O_{126} glycopeptide (see Fig. 2(b), (ii-iv)). Moreover, we were not able to detect acetylated Neu5Ac and Neu5Gc glycoforms of N₈₃-4Ant probably because sensitivity was not enough to detect these less abundant species. Table 2 shows the most abundant glycoforms detected for O_{126} and N₈₃-4Ant glycopeptides with and without reference compounds in the SL. The M_{theo} , M_{exp} , S/N values, RSD M_{exp} and av. errors are also illustrated in Table 2. As happened before for the rhEPO peptides (see previous section), glyco-form S/N values decreased in the presence of reference compounds. However, in contrast to the results obtained for the peptides of the digest, now the presence of the reference





Figure 2. CE/TOFMS analysis of the main (a) O_{126} and (b) N_{83} -4Ant glycopeptide glycoforms. (i) Sum of EIEs and (ii), (iii) and (iv) *m*/z spectra for the different glycoforms. (Sample: 1000 mg·L⁻¹ of digested rhEPO; injection: 15 s at 50 mbar; voltage: +18 kV; T: 25 °C BGE: 50 mM HAc and 50 mM HFor pH 2.2. Fragmentor voltage 270 V).

compounds in the SL allowed slightly better M_{exp} repeatability but had a detrimental effect on M_{exp} accuracy. Hence, the presence of reference compounds impedes glycopeptide detection without improving mass accuracy. For this reason, we continued our investigation without using reference compounds in the SL, and we recommend avoiding their use for the analysis of glycopeptides or peptides at low concentration.

As is well known, one of the main limitations of CE is the adsorption of the analytes in the fused-silica capillary inner surface. This phenomenon is especially important in analytes of peptide nature with a larger molecular mass such as glycopeptides, as they strongly interact with the negative charges of the silanols of the capillary wall. This adsorption usually provides low separation efficiency and poor reproducibility in terms of migration time and peak area. The use of coated capillaries is probably the best alternative to solve this negative effect.^[17] However, coatings are usually incompatible with MS detection since they bleed to some extent producing contamination of the mass spectrometer.^[3] In this work, with the aim of solving glycopeptide adsorption on the capillary wall avoiding the need to use coatings, two capillary washing procedures between analyses were evaluated: washing the capillary with BGE or with 1 M HAc before the BGE. Figure 3 shows, as an example, the results obtained with O_{126} glycopeptide glycoforms with both capillary washing procedures. As can be observed in the sum of EIEs of Fig. 3(a), BGE washing was not enough to avoid carry-over as one extra peak was detected at 16 min, which corresponded to a mixture of various O₁₂₆ glycopeptide glycoforms (O₁₂₆/0Neu5Ac, O₁₂₆/1Neu5Ac, O₁₂₆/2Neu5Ac). On the contrary, 1M HAc washing seemed to solve this problem, as the extra peak

		0	5 1 1	0,			. ,				
				WITH REFERENCE $(n=6)$				WITHOUT REFERENCE (n=6)			
	Glycopeptide glycoforms rhEPO (T) digest	M _{Theo} ^a (Da)	S/N	M _{exp} ^a (Da)	RSD M _{exp} (%)	Av. error (ppm) ^b	S/N	M _{exp} ^a (Da)	RSD M _{exp} (%)	Av. error (ppm) ^b	
O ₁₂₆	O ₁₂₆ / 0Neu5Ac	1829,8895	51,9	1829,8838	1,09 x10 ⁻⁴	3,09	45,6	1829,8852	2,35 x10 ⁻⁴	2,32	
	O ₁₂₆ / 1Neu5Ac	2120,9849	2077,1	2120,9786	5,67 x10 ⁻⁵	2,96	3096,3	2120,9818	1,03 x10 ⁻⁴	1,44	
	O ₁₂₆ / 2Neu5Ac	2412,0803	2078,9	2412,0754	1,16 x10 ⁻⁴	2,04	5805,3	2412,0795	2,27 x10 ⁻⁴	0,35	
N ₈₃ - 4Ant	N ₈₃ -4Ant/ 2Neu5Ac	5439,3280	37,8	5439,2871	5,88 x10 ⁻⁴	7,51	70,6	5439,3017	4,81 x10 ⁻⁴	4,83	
	N ₈₃ -4Ant/ 3Neu5Ac	5730,4234	528,4	5730,4003	1,24 x10 ⁻⁴	4,03	827,8	5730,4117	3,08 x10 ⁻⁴	2,05	
	N ₈₃ -4Ant/ 4Neu5Ac	6021,5188	1411,0	6021,4961	3,68 x10 ⁻⁴	3,78	2145,2	6021,4871	4,46 x10 ⁻³	5,27	
^a M _{Theo} ^b Avera	and M _{exp} : Mono ge error was calcu	isotopic mole ılated as: I(M	ecular ma _{exp} -M _{theo}	sses.) / M _{theo} l							

Table 2. Main O126 and N83-4Ant glycopeptide glycoforms detected from rhEPO (T)

O₁₂₆/1Neu5Ac **O₁₂₆** O₁₂₆/2Neu5Ac x10⁵ adsorption O₁₂₆/2Neu5Ac ž O₁₂₆/0Neu5Ac 0,6 0,4 **O**₁₂₆ adsorption O₁₂₆/1Neu5Ac O₁₂₆/0Neu5Ac Intensity (counts) b) 1M HAc capillary washing O₁₂₆/1Neu5Ac O₁₂₆/2Neu5Ac v10.4 1,4-1,2-O₁₂₆/2Neu5Ac 0,8 126/0Neu5Ac 0,6 0,4 0,2 1 Herry Mary 14,5 15 15,5 16 16,5 11.5 12 12.5 11 13.5 14 10 5 O₁₂₆/1Neu5Ac O₁₂₆/0Neu5Ac 10 11 12 13 14 15 16 17 18 19 20 7 8 9

a) BGE capillary washing



Time (min)





Figure 4. EIEs of the main (a) O_{126} and (b) N_{83} -4Ant glycopeptide glycoforms by CE/TOFMS. (Sample: 1000 mg/mL of protein digest; injection: 15 s at 50 mbar; voltage: +18 kV; T: 25 °C BGE: 50 mM HAc and 50 mM HFor pH 2.2. Fragmentor voltage 270 V).

completely disappeared (see Fig. 3(b)). Furthermore, %RSD of migration times improved from \sim 3% to \sim 0.5% confirming that 1 M HAc washing improved the repeatability of the method. Consequently, this capillary conditioning was considered the most appropriate for the analysis of glycopeptides by CE/TOFMS.

The established method allowed to detect O_{126} and N_{83} -4Ant from rhEPO tryptic digests. Nevertheless, we noticed these glycopeptides were susceptible to fragmentation. The most predominant fragmentation was the loss of Neu5Ac residues due to the lability of these sugar units. Figure 4 shows the EIEs of the main detected glycoforms of O_{126} and N_{83} -4Ant glycopeptides (Figs. 4(a) and 4(b), respectively). Each solid line corresponds to a different glycoform. As can be observed in Fig. 4(a), due to fragmentation, a small proportion of O_{126} /0Neu5Ac appeared at the same migration time

as O₁₂₆/1Neu5Ac (~12.5 min). Similarly, the glycoforms with 0Neu5Ac and 1Neu5Ac units were detected at the migration time corresponding to $O_{126}/2Neu5Ac$ (~15.5 min). That fragmentation behavior was also observed for N₈₃-4Ant glycopeptide glycoforms (Fig. 4(b)). Thus, glycoforms with 3Neu5Ac and 2Neu5Ac were detected with N₈₃-4Ant/ 4Neu5Ac, while the glycoform with 2Neu5Ac was detected with N₈₃-4Ant/3Neu5Ac. Sugar fragmentation seemed to be produced during the ionization process and not during the electrophoretic separation, as the precursor and product ions appeared at the same migration time regardless of their electrophoretic mobilities. Following the same argument, other product ions could be observed apart from those resulting from Neu5Ac fragmentation. By way of example, Table 3 shows the detected product ions coming from O₁₂₆/2Neu5Ac and N_{83} -4Ant/4Neu5Ac precursor ions. In the case of O_{126} /



	Glycopeptide glycoform (Parent Ion)	Observed Fragmentation ^a (Product Ions)
O ₁₂₆	Pep+HexNAc+Hex+2Neu5Ac	Pep+HexNAc+Hex+Neu5Ac Pep HexNAc+1Neu5Ac Pep+HexNAc+Hex Pep+HexNAc Pen
N ₈₃ -4Ant	Pep+6HexNAc+7Hex+1Fuc+4Neu5Ac	Pep+6HexNAc+7Hex+1Fuc+3Neu5Ac Pep+6HexNAc+7Hex+1Fuc+2Neu5Ac Pep+5HexNAc+6Hex+1Fuc+3Neu5Ac Pep+4HexNAc+5Hex+1Fuc+2Neu5Ac Pep+1HexNAc Pep+1HexNAc Pep



Figure 5. Percentage of peak area (%A) (n=6) obtained for (a) $O_{126}/2Neu5Ac$ and (b) N_{83} -4Ant/4Neu5Ac precursor ions and their corresponding product ions at the different fragmentor voltage values by CE/TOFMS. Error bars show the standard deviations (s). S/N values for each precursor ion are also indicated.

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2Neu5Ac, we observed all possible fragmentations: Desialylations but also the loss of Hex and HexNAc units, leaving O_{126} peptide free of sugars. Some authors described the presence of the non-glycosylated O_{126} in rhEPO samples by MALDI-MS.^[14] However, our results suggest the peptide detected by Stübiger *et al.*^[14] probably originated from the fragmentation of the glycopeptides taking into account that intact glycoproteins and glycopeptides may undergo fragmentation easily by MALDI-MS depending on the detection conditions.^[8,18] As can be observed in Table 3, N₈₃-4Ant/ 4Neu5Ac showed more product ions than $O_{126}/2Neu5Ac$ due to the higher sugar content of this glycopeptide.

Tandem mass spectrometry is widely used for the characterization of glycans and glycopeptides as fragmentation patterns may reveal finer details of glycan structure to completely characterize the various post-translational modifications of a glycoprotein.^[19–21] However, in our case, glycopeptide fragmentation must be prevented as the aim was the comprehensive detection of the intact glycopeptide glycoforms of the protein digest to achieve a reliable characterization of the glycoprotein. Hence, there can be no doubts as to whether the detected glycoforms were present in the native glycopeptide glycoforms.

One explanation for the observed fragmentation could be the acidic conditions of the BGE and SL as, for example, sialic acids (Neu5Ac or Neu5Gc) can be hydrolyzed under acidic conditions. Using the acidic BGE of 50 mM HAc and 50 mM HFor (pH 2.2), we tested different percentages of HFor in the SL: 0.01, 0.05 and 0.1% v/v of HFor. The percentages of peak area (%A), measured from the extracted ion electropherogram (EIE), of each precursor and their corresponding product ions were selected to evaluate the extent of O126/2Neu5Ac and N83-4Ant/4Neu5Ac glycopeptide fragmentation. The percentage of peak area (%A) obtained for the product ions of the glycopeptides remained practically constant at the different percentages of HFor. The SL with 0.1% of HFor was discarded because it provided lower sensitivity due to the increase in the background signal. We selected 0.05% v/v of HFor as the optimum percentage since migration time and S/N values were more reproducible than with 0.01% v/v of HFor. As decreasing the acidity of the SL did not avoid fragmentation, we explored the use of a basic BGE (50 mM NH₄Ac, pH 8). However, the results were worse than those obtained with the acidic BGE. Glycopeptides were poorly separated, sensitivity extremely decreased and fragmentation of O126/2Neu5Ac and N83-4Ant/4Neu5Ac was also observed.

Once we had investigated if the acidic conditions were responsible for the glycopeptide fragmentation, we considered the influence of the selected TOF parameters. The fragmentor voltage is the MS parameter that accelerates the ions of interest towards the detector. However, its value has been described to be directly related to in-source collision-induced dissociation (ISCID) of neuropeptides, pesticides and antibiotics.^[12,22,23] Therefore, six fragmentor voltage values were evaluated: 160, 190, 215, 240, 270 and 325 V. Figure 5 shows the percentage of peak area (%A) of O₁₂₆/2Neu5Ac and N₈₃-4Ant/4Neu5Ac precursor ions and their corresponding product ions at the different fragmentor voltage values (Figs. 5 (a) and 5(b), respectively). Error bars show the standard deviations obtained for %A (n=6). Moreover, S/N values

obtained for each precursor ion at the different fragmentor voltages are also illustrated in Fig. 5. Although we observed a slight fragmentation of $O_{126}/2Neu5Ac$ mainly due to the loss of Neu5Ac residues, the abundance of the precursor ion at low fragmentor voltage values (160-190 V) was the highest (near 100%) (Figs. 5(a) and 5(b)). As the value increased, fragmentation of the precursor ion was promoted, increasing the percentage of the product ions (see Fig. 5). This was also the reason why in general S/N values of the precursor ion were lower at higher fragmentor voltage values. The fragmentor voltage 190 V was selected as the optimum fragmentor voltage value for O₁₂₆/2Neu5Ac since it provided higher S/N values than 160 V and fragmentation was rather low in comparison with that obtained at 215 V. Regarding N₈₃-4Ant/ 4Neu5Ac, no fragmentation was observed at 160-190 V (see Fig. 5(b)) which confirmed the higher stability of N_{83} -4Ant compared to O₁₂₆ glycopeptides as other authors suggested.^[15] An optimal fragmentor voltage of 190 V was also selected for N_{83} -4Ant/4Neu5Ac as it gave better S/N values than 160 V. Hence, although $N_{83}\mbox{-}4\mbox{Ant}/4\mbox{Neu5Ac}$ and $O_{126}/$ 2Neu5Ac glycopeptides showed a great number of structural differences, we were able to select an optimum fragmentor voltage value that minimized fragmentation for both glycopeptides. These conditions may be useful to obtain a reliable glycopeptide map of rhEPO and other glycoproteins.

CONCLUSIONS

A CE/TOFMS method using a volatile BGE of 50 mM HAc and 50 mM HFor at pH 2.2 was developed for the analysis of glycopeptides present in rhEPO tryptic digests. A capillary conditioning consisting in 1 M HAc washing was selected to prevent carry over due to glycopeptide adsorption on the capillary wall. It was demonstrated that the presence of reference compounds in the SL had a detrimental effect on the detection of the peptides and glycopeptides of the protein digest. Furthermore, we proved the importance of the selection of an appropriate fragmentor voltage value for a unambiguous glycopeptide detection and quantification by CE/TOFMS. We were able to select the same optimum fragmentor voltage value for N₈₃-4Ant and O₁₂₆ despite the great structural differences between both glycopeptides. These conditions may be regarded as an excellent starting point to obtain reliable glycopeptide maps of complex glycoprotein enzymatic digests by CE/TOFMS.

Acknowledgements

Part of this work was supported by the Spanish Ministry of Science and Innovation (CTQ2008-00507/BQU).

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