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# High-resolution glycoform profiling of intact therapeutic proteins by hydrophilic interaction chromatography-mass spectrometry

Elena Domínguez-Vega[a,1], Sara Tengattini[b], Claudia Peintner[a], Jordy van Angeren[a], Caterina Temporini[b], Rob Haselberg[a], Gabriella Massolini[b], Govert W. Somsen[a,*]

[a] Division of BioAnalytical Chemistry, Department of Chemistry and Pharmaceutical Sciences, Vrije Universiteit Amsterdam, de Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
[b] Department of Drug Sciences, University of Pavia, via Taramelli 12, I-27100 Pavia, Italy

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ABSTRACT

Glycosylation is considered a critical quality attribute of therapeutic proteins. Protein heterogeneity introduced by glycosylation includes differences in the nature, number and position of the glycans. Whereas analysis of released glycans and glycopeptides provides information about the composition and/or position of the glycan, intact glycoprotein analysis allows assignment of individual proteoforms and co-occurring modifications. Yet, resolving protein glycoforms at the intact level is challenging. We have explored the capacity of hydrophilic liquid chromatography-mass spectrometry (HILIC-MS) for assessing glycosylation patterns of intact pharmaceutical proteins by analyzing the complex glycoproteins interferon-beta-1a (rhIFN-β − 1a) and recombinant human erythropoietin (rhEPO). Efficient glycoform separation was achieved using a superficially-porous amide HILIC stationary phase and trifluoroacetic acid (TFA) as eluent additive. In-source collision-induced dissociation proved to be very useful to minimize protein-signal suppression effects by TFA. Direct injection of therapeutic proteins in aqueous formulation was possible without causing extra band dispersion, provided that the sample injection volume was not larger than 2 µL. HILIC-MS of rhIFN-β − 1a and rhEPO allowed the assignment of, respectively, 15 and 51 glycoform compositions, next to a variety of posttranslational modifications, such as succinimide, oxidation and N-terminal methionine-loss products. MS-based assignments showed that neutral glycan units significantly contributed to glycoform separation, whereas terminal sialic acids only had a marginal effect on HILIC retention. Comparisons of HILIC-MS with the selectivity provided by capillary electrophoresis-MS for the same glycoproteins, revealed a remarkable complementarity of the techniques. Finally it was demonstrated that by replacing TFA for difluoroacetic acid, peak resolution somewhat decreased, but rhEPO glycoforms with relative abundances below 1% could be detected by HILIC-MS, increasing the overall rhEPO glycoform coverage to 72.

## 1. Introduction

Glycosylation is one of the most common posttranslational modifications (PTMs) of proteins. Glycosylation involves the covalent attachment of oligosaccharides (glycans) to the amino acid backbone of a protein, in particular to serine/threonine (O-glycosylation) or asparagine (N-glycosylation) residues. This glycan decoration has a major impact on the biological function of the protein and may affect protein stability, solubility, antigenicity, folding and serum half-life [1,2]. A large part of the proteins exploited today by the biopharmaceutical industry displays oligosaccharides. Protein glycosylation is a complex process that involves various enzymes and substrates, and depends on the host organism, production cell line, and culture conditions [3,4]. The glycosylation pattern is considered a critical quality attribute of recombinant pharmaceutical proteins and should be carefully monitored to ensure quality, safety, and efficacy of the biopharmaceutical product.

Protein heterogeneity introduced by glycosylation includes differences in the nature and structure of the attached oligosaccharides, and the number and position of the glycans [5]. Common methods to study glycosylation involve glycan release or protein digestion, followed by analysis of the resulting glycans or glycopeptides, respectively [5,6]. These methods offer detailed information about the sites of glycosylation and/or the carbohydrate composition, however, the number of

* Corresponding author.
*E-mail address:* g.w.somsen@vu.nl (G.W. Somsen).
[1] Current address, Center for Proteomics and Metabolomics, Leiden University Medical Center, Postbus 9600, 2300 RC Leiden, The Netherlands.

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attached glycans per protein molecule or molecule specific combinations with other PTMs are not revealed. Analysis at the intact protein level offers complementary structural information and allows assignment of individual proteoforms, which often include combinations of multiple modifications. Moreover, sample preparation can be limited, if not totally omitted, preventing undesired modifications induced by enzymatic treatments as well as reducing total analysis time [7].

Electrospray ionization mass spectrometry (ESI-MS) enables the precise mass determination and characterization of intact (glyco)proteins. However, biopharmaceuticals frequently comprise a large number of glycoforms, differing in number and nature of the attached glycans, which may not be distinguished consistently by MS only. Separation prior to MS detection often is essential to achieve reliable assignment of intact glycoproteins and detection of low abundant glycoforms. Nevertheless, conventional protein separation techniques generally show poor compatibility with MS (as e.g. hydrophobic interaction chromatography and ion-exchange chromatography) or lack the selectivity to resolve glycoforms (as e.g. reversed-phase liquid chromatography).

Hydrophilic interaction chromatography-mass spectrometry (HILIC-MS) has shown highly useful for the analysis of a wide range of polar compounds, including metabolites and peptides. In the context of glycoprotein characterization, HILIC-MS has been widely used for the determination of released glycans and glycopeptides. So far, however, application of HILIC-MS for the analysis of intact proteins has been quite limited, most probably due to (supposed) issues with protein solubility, adsorption and band dispersion using conventional HILIC stationary phases [8–12]. Following pioneering studies indicating the possibilities of HILIC for intact protein analysis [13–15], lately, new superficially-porous and wide-pore neutral amide-bonded stationary phases have shown great potential for HILIC-MS of intact glycosylated proteins [16–19]. Employing ion-pairing agents in the mobile phase, glycoprotein retention in HILIC was made primarily governed by carbohydrate content, while still allowing MS detection. Unprecedented glycoform resolution, which was based on the number and size of the attached glycans, was demonstrated for semi-synthetic glycoproteins and ribonuclease B (RNase B) [13,19]. Along the same lines, D'Atri et al. showed the potential of HILIC-MS for the glycoform profiling of Fc fragments of therapeutic monoclonal antibodies obtained after IdeS-digestion, demonstrating the usefulness of the approach for the comparison of biosimilar antibodies and their originators [16,17].

In the present work, we explored the capacity of HILIC-MS for assessing glycosylation patterns of intact pharmaceutical proteins. For that purpose, we first studied in-source conditions to minimize suppression of glycoprotein signals by the applied ion-pair reagent. In addition, the possibility of direct injection of aqueous glycoprotein samples was examined. The utility of the developed HILIC-MS method for detailed profiling of intact pharmaceutical glycoproteins was studied by the analysis of recombinant human interferon-beta-1a (rhIFN-β − 1a) and recombinant human erythropoietin (rhEPO), which comprise large numbers of glycoforms, but also other PTMs. MS-based assignment of glycoforms helped to reveal the relative contribution of specific glycans to HILIC retention, allowing a practical comparison with the selectivity previously obtained with capillary electrophoresis-mass spectrometry (CE-MS) for the same glycoproteins.

## 2. Experimental

### 2.1. Chemicals and samples

Acetic acid (AA), difluoroacetic acid (DFA), ammonium acetate and ammonium formate were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid (FA) was obtained from Merck (Darmstadt, Germany). Biosolve B.V (Valkenswaard, The Netherlands) supplied acetonitrile (ACN) and trifluoroacetic acid (TFA). Ribonuclease A and ribonuclease B (RNase A and RNase B from bovine pancreas) were obtained from Sigma-Aldrich (Steinheim, Germany). Recombinant human interferon-β − 1a (rhIFN-β − 1a; Avonex®) from Biogen Idec (Cambridge, MA) was collected from prefilled syringes. The European Pharmacopeia provided lyophilized rhEPO (chinese hamster ovary cell line) as a biological reference product (Batch 4; 13 000 IU per vial).

Standard stock solutions of RNase B were prepared in pure water at a concentration of 2 mg/mL and then diluted with ACN/water to obtain working solutions of 0.5 mg/mL in ACN-water (50:50, v/v). Lyophilized rhEPO was reconstituted in purified water in order to reach a final concentration of 2.5 μg/μL. The rhIFN-β − 1a (0.25 μg/μL) were analysed as obtained, that is, without any sample pretreatment.

### 2.2. HILIC

Chromatographic separations were performed using an Agilent Technologies HPLC series 1200 system (Palo Alto, CA, USA), equipped with a mobile-phase online degasser, quaternary pump, autosampler, column thermostated compartment, and diode array detector. For data acquisition and analysis, ChemStation software version Rev. B.04.01 was used in a Microsoft Windows XP environment. An AdvanceBio Glycan Map (150 × 2.1 mm; 2.7 μm) column from Agilent Technologies was used. The injection volume was 2 μL and the flow rate was 0.5 mL/min. The column temperature was 40, 50 and 60 °C for EPO, RNase B and rhIFN-β − 1a analyses, respectively. The final mobile phases were composed of ACN (A) and water (B) both containing 0.1% (v/v) TFA for RNase B and rhIFN-β − 1a or 0.1% DFA (v/v) for EPO. Gradient elution conditions were optimized for each protein and as follows: for RNase B, from 28% to 38% B in 20 min followed by 38% B for 10 min; for rhIFN-β − 1a, from 25% to 30% B in 10 min followed by 30% B for 10 min; for EPO, from 28% to 33% B in 7 min and from 33% to 38% in 18 or 28 min followed by 38% B for 10 min. UV absorbance was monitored at 214 nm.

### 2.3. Mass spectrometry

Mass spectrometric detection was carried out using a maXis HD ultra-high resolution quadrupole time-of-flight (qTOF) mass spectrometer from Bruker Daltonics (Bremen, Germany) equipped with an ESI source. The mass spectrometer was operated in positive-ion mode with an electrospray voltage of 4.5 kV. The nebulizer and drying gas conditions were 1.0 bar and 8.0 L/min nitrogen at 200 °C, respectively. Quadrupole ion energy and collision cell energy were set at 5.0 and 15.0 eV, respectively. Transfer and pre plus storage times were 190.0 and 20.0 μs, respectively. In source collision induced dissociation (ISCD) was set at 120 eV in order to dissociate protein-TFA adducts formed during ESI. The monitored $m/z$ range was 250 − 5000 $m/z$. Extracted-ion chromatograms (EICs) were obtained with an extraction window of ± 0.5 $m/z$ using the smooth option of the software (Gaussian at 1 point). Molecular mass determinations of proteins were performed using the "Maximum Entropy" utility of the Data Analysis software.

## 3. Results and discussion

We have evaluated the use of HILIC-MS for the glycoform profiling of intact therapeutic proteins. For this purpose, the pharmaceutical glycoproteins rhIFN-β − 1a and rhEPO, which represent different glycosylation complexity, were selected. rhIFN-β − 1a is a therapeutic protein of 166 amino acids containing one N-glycosylation site at Asn-80 carrying a variety of complex type glycans. rhEPO consists of 165 amino acids with three N-glycosylation sites (Asn-24, Asn-38, Asn-83) and one O-glycosylation site (Ser-126), leading to an extensive glycoform heterogeneity. RNase B was used as test glycoprotein during initial optimization. RNase B has a molecular weight of 15 kDa and contains a single N-glycosylation site at Asn-34 giving rise to five oligomannose glycoforms differing in the number (5 − 9) of mannose residues.

### 3.1. HILIC-MS conditions for intact glycoprotein analysis

Addition of TFA to the eluent has demonstrated to be essential for achieving good glycoprotein resolution by amide HILIC [13,17–19]. For instance, using ammonium formate at pH 3.5 as mobile-phase additive, elution of RNase B requires high percentages of water and glycoform separation was poor (Fig. S1a), while using ammonium formate at pH 6.0 elution of the protein was not observed. Applying formic acid as eluent additive, the glycoprotein shows less retention and elutes at a much lower percentage of water, but the RNase B glycoforms are still overlapping (Fig. S1b). When TFA is part of the mobile phase, baseline separation of all glycoforms of RNase B is achieved (Fig. S1c). In presence of TFA, the acidic amino acid residues of the glycoprotein are fully protonated (i.e. neutral), while the negatively-charged TFA forms ion pairs with the protonated basic residues, overall increasing protein hydrophobicity and minimizing potential ionic interactions of the protein [14,17,18]. Under these conditions, glycoprotein separation is mainly governed by the carbohydrate content, with glycoform retention raising with increasing size and number of the attached glycans [13,19].

TFA is volatile, however, it still lowers protein MS signals and, therefore, reduces detection sensitivity [20–22]. The main reason for signal suppression is the strong gas-phase affinity of TFA for positively-charged proteins. For example, during HILIC-MS of RNase B using a mobile phase containing 0.05% TFA (Fig. 1A)., each protein charge state showed a number of TFA adducts, reducing the absolute signal intensity (Fig. 1B). Dissociation of glycoprotein-TFA adducts can be achieved by applying ISCID. Employing an ISCID energy of 120 eV resulted in entire removal of the TFA molecules from the glycoprotein ion, leading to a single signal per charge state with an increased intensity (Fig. 1B3). The obtained mass spectra showed that charge-state distribution remained unaffected by the use of ISCID, indicating that the adducts dissociate to yield the neutral acid, as also previously described [23]. The ISCID energy could be increased up to 200 eV without inducing significant fragmentation of the glycoprotein and decrease of signal intensities (Fig. 1B4). An ISCID energy of 120 eV was applied in further studies, ensuring proper intact protein detection without causing in-source degradation.

Biopharmaceuticals are often formulated in aqueous buffer solutions. In conventional HILIC the sample solvent commonly is chosen similar to the composition of the starting eluent in order to prevent peak deformation and broadening. Still, due to solubility problems, high percentages of ACN cannot be used for intact protein analysis. When glycoproteins were dissolved in 1:1 (v/v) ACN-water and analysed by HILIC-MS, good peak shapes were obtained. However, in this solvent, our test proteins sometimes appeared not to be stable over time and protein precipitation and/or degradation (i.e. appearance of additional peaks) occasionally was observed. Fortunately, it appeared possible to analyse glycoproteins dissolved in 100% water as long as a relatively small injection volume was applied. Up to 2 µL of aqueous sample could be injected without causing extra band broadening or peak deformation, which is in good agreement with observations made during HILIC-MS of mAb fragments [17]. Using an injection volume of 2 µL, HILIC-MS repeatability was assessed by performing six consecutive injections of RNase B. RSDs of 0.4% and 1.6% were obtained for, respectively, retention time and peak area of the most abundant glycoform, indicating stable performance without protein losses.

### 3.2. Characterization of intact proteoforms of rhIFN-β − 1a

Described glycoforms of rhIFN-β − 1a are mostly mono-fucosylated (Fuc) bi-, tri- and tetra-antennary structures comprising hexoses (Hex; mannose or galactose), N-acetylhexosamines (HexNAc; N-acetylgalactosamine) and sialic acids (SiA). Additional PTMs of rhIFN-β − 1a include deamidation, succinimide intermediates, and oxidation [24,25].

Aqueous formulated rhIFN-β − 1a (Avonex; 0.25 µg/µL) was directly analysed by HILIC without any sample pretreatment. A column temperature of 60 °C and a relatively shallow ACN-water gradient (25–30% water in 10 min) provided optimal separation of rhIFN-β − 1a proteoforms. Increasing the concentration of TFA up to 0.1% resulted in better glycoform resolution. Using these conditions, HILIC-MS of rhIFN-β − 1a revealed a profile of peaks eluting between 3 and 10 min (Fig. 2A), and good quality mass spectra were obtained for the respective bands. The deconvoluted mass spectrum of the main peak at 5.5 min presented a mass of 22377.1 Da, which could be assigned to the rhIFN-β − 1a protein backbone carrying one fucosylated disialylated biantennary glycan structure comprising five Hex and four HexHexNAc units (i.e. $Hex_5HexNAc_4Fuc_1SiA_2$). This composition for the most abundant glycoform is in good agreement with previous analytical

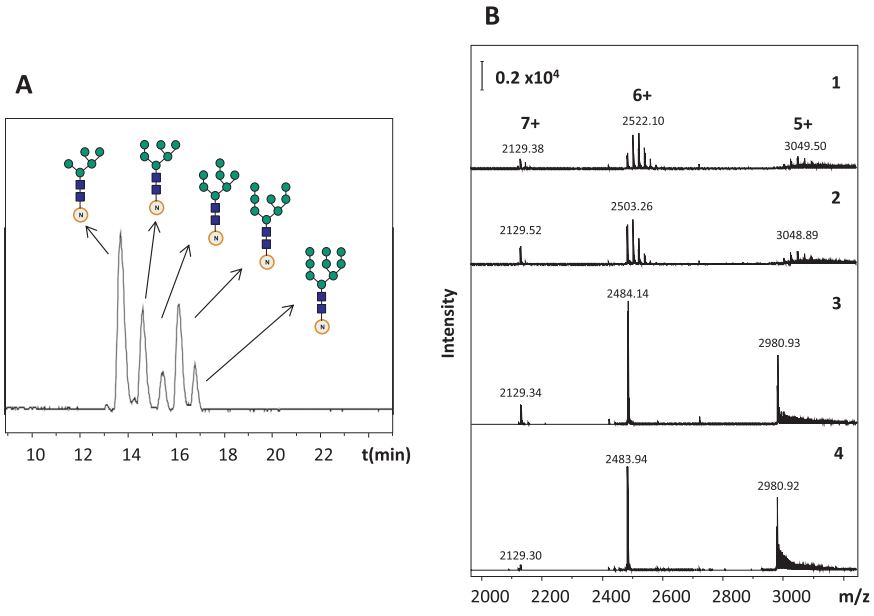


**Fig. 1.** HILIC-MS of RNase B. (A) Base-peak chromatogram; (B) Mass spectra recorded for the main glycoform. Applied ISCD energy, (1) 0, (2) 60, (3) 120 or (4) 200 eV. Further conditions, see Experimental section. HILIC gradient, 28–38% B in 20 min followed by 38% B for 10 min. Further conditions, see Experimental section.

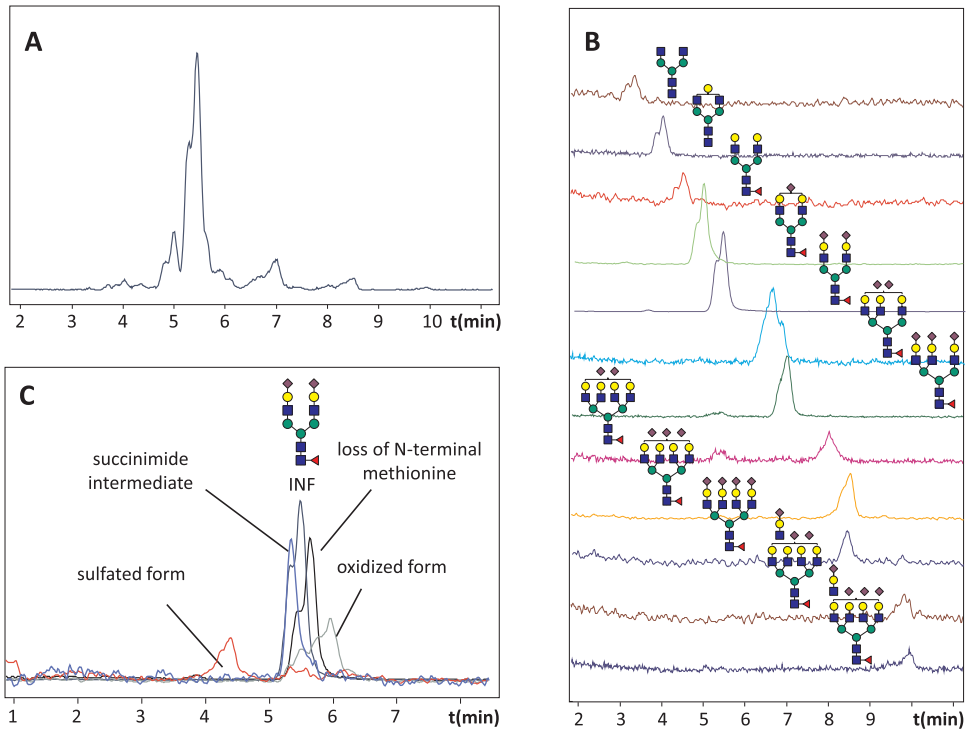


**Fig. 2.** HILIC-MS of rhIFN-β-1a. (A) Base-peak chromatogram; (B) Extracted-ion chromatograms of the detected glycoforms with tentative glycan assignment; (C) Extracted-ion chromatograms of PTMs of the most abundant glycoform. HILIC gradient, from 25% to 30% B in 10 min followed by 30% B for 10 min. Further conditions, see Experimental section.

**Table 1**
Retention time, molecular mass ($M_w$), N-glycan composition and PTM for rhIFN-β − 1a proteoforms as observed with HILIC-MS.

| retention time (min) | $M_w$ (Da) | assigned glycan composition | assigned PTM |
|---|---|---|---|
| 3.4 | 21,321.8 | $Hex_3HexNAc_4Fuc_0SiA_0$ | – |
| 4.0 | 21,483.6 | $Hex_4HexNAc_4Fuc_0SiA_0$ | – |
| 4.4 | 22,457.1 | $Hex_5HexNAc_4Fuc_1SiA_2$ | SG |
| 4.5 | 21,792.7 | $Hex_5HexNAc_4Fuc_1SiA_0$ | – |
| 4.9 | 22,085.2 | $Hex_5HexNAc_4Fuc_1SiA_1$ | – |
| 5.1 | 21,954.1 | $Hex_5HexNAc_4Fuc_1SiA_1$ | Des − 1 |
| 5.4 | 22,360.0 | $Hex_5HexNAc_4Fuc_1SiA_2$ | Succ |
| 5.5 | 22,377.1 | $Hex_5HexNAc_4Fuc_1SiA_2$ | – |
| 5.6 | 22,246.0 | $Hex_5HexNAc_4Fuc_1SiA_2$ | Des − 1 |
| 5.9 | 22,668.1 | $Hex_5HexNAc_4Fuc_1SiA_3$ | – |
| 6.0 | 22,393.1 | $Hex_5HexNAc_4Fuc_1SiA_2$ | Ox |
| 6.1 | 22,523.1 | $Hex_5HexNAc_4Fuc_2SiA_2$ | – |
| 6.8 | 22,742.2 | $Hex_6HexNAc_5Fuc_1SiA_2$ | – |
| 6.9 | 22,611.0 | $Hex_6HexNAc_5Fuc_1SiA_2$ | Des − 1 |
| 7.0 | 23,033.2 | $Hex_6HexNAc_5Fuc_1SiA_3$ | – |
| 7.2 | 22,902.4 | $Hex_6HexNAc_5Fuc_1SiA_3$ | Des − 1 |
| 7.4 | 23,049.2 | $Hex_6HexNAc_5Fuc_1SiA_3$ | Ox |
| 8.1 | 23,107.2 | $Hex_7HexNAc_6Fuc_1SiA_2$ | – |
| 8.4 | 23,398.4 | $Hex_7HexNAc_6Fuc_1SiA_3$ | – |
| 8.4 | 23,689.4 | $Hex_7HexNAc_6Fuc_1SiA_4$ | – |
| 8.5 | 23,267.4 | $Hex_7HexNAc_6Fuc_1SiA_3$ | Des − 1 |
| 9.8 | 23,763.4 | $Hex_8HexNAc_7Fuc_1SiA_3$ | – |
| 9.9 | 24,054.8 | $Hex_8HexNAc_7Fuc_1SiA_4$ | – |
| 10.7 | 24,129.4 | $Hex_9HexNAc_8Fuc_1SiA_3$ | – |

SG, sulfated glycan.
Des-1, loss of N-terminal methionine.
Succ, succinimide intermediate.
Ox, oxidation.

works on rhIFN-β − 1a [24,25]. Deconvoluted mass spectra were constructed for 0.5-min time intervals throughout the entire chromatogram. Eluting before and after the main peak, various glycoforms of rhIFN-β − 1a were detected which, based on their determined mass, differed in the number of Fuc (146 Da), Hex (162 Da), HexNAc (203 Da) and SiA (291 Da) units (Fig. 2B). As can be concluded from the figure, glycoform retention overall increased with the size of the glycan. Addition of Hex and HexHexNAc units to the glycan chain resulted in a bigger retention shift than addition of a SiA. Especially for glycans bigger in size (e.g. $Hex_7HexNAc_6Fuc_1$ and $Hex_8HexNAc_7Fuc_1$), the presence of SiA hardly affected the retention of the glycoform. Proteoforms of rhIFN-β − 1a that were assigned based on the obtained HILIC-MS data are summarized in Table 1.

Next to glycoforms, PTMs such as oxidation, succcinimide intermediates and loss of N-terminal methionine were observed. Fig. 2C shows the EICs of the proteoforms detected for the main glycoform ($Hex_5HexNAc_4Fuc_1SiA_2$). The loss of N-terminal methionine (a hydrophobic amino acid) as well as methionine oxidation resulted in an increase of HILIC retention, which both might be explained by a relative increase of protein polarity. The observed succinimide intermediate can be formed during the process of protein deamidation. Succinmidation lowers protein polarity, and indeed the succinimide intermediate showed a slightly lower HILIC retention than the unmodified glycoform. Deamidation has been reported to be a major modification of therapeutic rhIFN-β − 1a and most probably was induced deliberately to increase half-life and potency [26]. Closer inspection of the HILIC-MS EICs of rhIFN-β − 1a (Fig. 2B and C) showed that, with the exception of the succinimide intermediate, the proteoform peaks exhibit an unresolved shoulder. We presume the shoulder is caused by the deamidated form, which is partially resolved from the non-deamidated form. Deamidation gives rise to a protein mass increase of just 1 Da and, therefore, deamidated/non-deamidated proteoforms cannot be reliably distinguished by MS only. For the most abundant glycoform, a variant with a deconvoluted mass of 22,457.0 Da (+ 79.9 Da) was detected (Fig. 2C) at 4.4 min, which may be ascribed to protein phosphorylation, sulfation or to the presence of a sulfated glycan. A previous CE-MS study on rhIFN-β − 1a revealed a proteoform with the same mass which, following exoglycosidase treatment, was assigned to a sulfation of the glycan [24]. Apparently this modification leads to a decrease of HILIC retention.

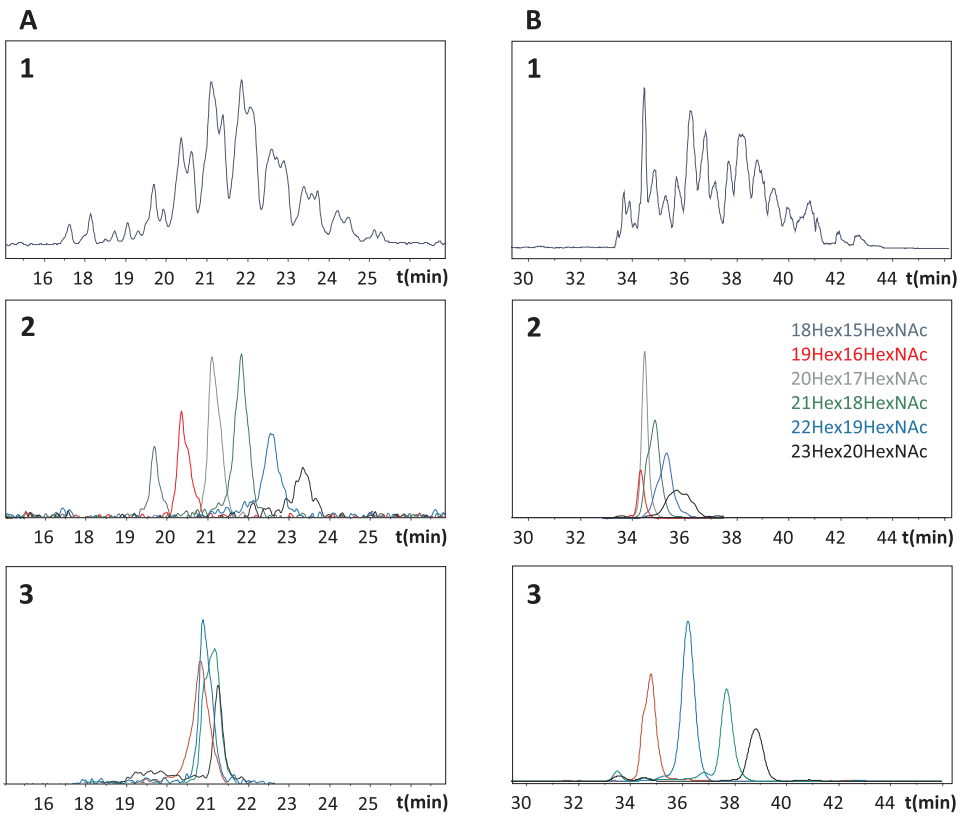


**Fig. 3.** (A) HILIC-MS and (B) CE-MS of rhEPO. (1) Base-peak chromatogram/electropherogram; (2) Extracted-ion chromatograms/electropherograms of glycoforms with overall glycan composition of $Hex_{19–24}HexNAc_{16–21}Fuc_3SiA_{10}$; (3) Extracted-ion chromatograms/electropherograms of glycoforms with overall glycan composition of $Hex_{21}HexNAc_{18}Fuc_3SiA_{10–13}$. HILIC gradient, 28–33% B in 7 min and from 33% to 40% in 18 min followed by 40% B for 10 min. Further HILIC-MS conditions, see Experimental section; CE-MS conditions, see Ref [25].

### 3.3. Glycoform profiling of rhEPO

Aqueous rhEPO solutions were directly analysed by HILIC using an ACN-water gradient (28–40% water in 35 min) and a column temperature of 40 °C. Increasing the concentration of TFA in the mobile phase from 0.05% to 0.1% resulted in a slightly better resolution of the rhEPO glycoforms. HILIC-MS analysis of rhEPO provided a wide pattern of partially resolved peaks eluting between 16 and 26 min (Fig. 3A1). The deconvoluted mass spectrum of the most intense band (21.7–22.2 min) revealed a main mass of 29888 Da (Fig. S2B), which based on previous reports could be assigned to rhEPO molecules comprising an overall glycan composition of $Hex_{22}HexNAc_{19}Fuc_3SiA_{19}$ [25,27,28]. Deconvoluted mass spectra were generated throughout the entire peak profile and the masses of the main protein bands were listed (Table 2). Using these masses, overall, 51 distinct glycoform compositions could be assigned that differed in number of HexHexNAc or SiA residues. Glycoforms eluting between 19 and 26 min contained three Fuc residues and consistently comprised three Hex residues more than HexNAc residues, which indicates that for these glycoforms the three N-glycosylation sites are occupied. Based on observed masses, the minor components with retention times of 16–19 min were assigned to glycoforms containing two Fuc residues and always two Hex more than HexNAc residues, suggesting that these glycoforms carry only two N-glycans.

The deconvoluted mass spectra obtained for all the rhEPO glycoforms typically exhibited several signals differing by 291 Da (i.e. one SiA residue) (Fig. S2B). This would suggest that SiA residues do not significantly add to the HILIC separation of the glycoforms. The mains peaks discerned in the base peak chromatogram appeared to show a constant mass difference of 365 Da corresponding to HexHexNAc (Fig. S2A). In order to better appreciate the rhEPO-glycoform resolution obtained by HILIC-MS, EICs for glycoforms containing three Fuc and 10 SiA residues, but with a varying number of HexHexNAc units were constructed (Fig. 3A2). Addition of one HexHexNAc appeared to result in an increase of the rhEPO glycoform retention time of about 0.9 min, underlining the strong contribution of neutral sugar units on the HILIC retention. The EICs for glycoforms containing a constant $Hex_{21}HexNAc_{18}Fuc_3$ composition, but a varying number of SiAs are depicted in Fig. 3A3. SiA indeed contributes to HILIC glycoform separation, but only quite marginally with an increase in retention time of about 0.2 min per added SiA residue. Interestingly, this result reveals a remarkable complementarity of HILIC-MS with respect to glycoform selectivity as obtained by CE-MS [25]. Fig. 3B shows the base peak electropherogram (3B1) and extracted-ion electropherograms (3B2 and 3B3) obtained for a rhEPO sample and selected glycoforms as previously obtained with CE-MS [25]. In CE-MS, the separation is evidently dominated by the SiA content of the glycoforms, whereas the number of HexHexNAc residues only moderately contributes to differences in glycoform migration times. Indeed, addition of SiA to a rhEPO glycoform involves a change of the overall protein charge, which has a more substantial effect on its electrophoretic mobility than the relatively small increase of protein mass/size induced by addition of HexHexNAc units. Hence, both HILIC-MS and CE-MS show very useful for intact glycoform profiling, exhibiting complementary separation selectivities towards neutral and charged glycan content, respectively.

Diglycosylated variants of rhEPO as assigned by HILIC-MS, were previously not found with CE-MS, probably because they were not separated from the other major glycoforms. On the other hand, the highly favorable mass sensitivity of the applied CE-MS method and its ability to efficiently separate sialoforms, allowed detection of several minor glycoforms. In an attempt to improve the sensitivity of HILIC-MS for intact glycoproteins, we tested difluoroacetic acid (DFA) as eluent additive replacing TFA. DFA has recently been proposed as ion-pairing agent in RPLC-MS [21,29], exhibiting similar chromatographic-performance enhancing effects as TFA, but causing less ionization suppression in ESI-MS detection. HILIC-MS of RNase B employing DFA as eluent additive was carried out and compared with HILIC-MS using TFA (Fig. 4 and S3). With 0.1% DFA glycoform peak areas were more than threefold higher than obtained with 0.1% TFA. However, with DFA, peak widths were larger and plate numbers lower, resulting in a lower

**Table 2**
Molecular mass and assigned glycan composition of rhEPO glycoforms observed with HILIC-MS employing TFA or DFA as mobile phase additive. Shaded masses are observed using TFA or DFA as additive. Masses printed *italic* were only observed using DFA.

| | $SiA_7$ | $SiA_8$ | $SiA_9$ | $SiA_{10}$ | $SiA_{11}$ | $SiA_{12}$ | $SiA_{13}$ | $SiA_{14}$ | $SiA_{15}$ |
|---|---|---|---|---|---|---|---|---|---|
| $Hex_{15}HexNAc_{13}Fuc_2$ | *25641.4* | 25933.3 | 26223.6 | 26515.3 | | | | | |
| $Hex_{16}HexNAc_{14}Fuc_2$ | | 26297.1 | 26588.6 | 26880.6 | | | | | |
| $Hex_{17}HexNAc_{15}Fuc_2$ | | 26663.9 | 26954.9 | 27245.9 | | | | | |
| $Hex_{18}HexNAc_{16}Fuc_2$ | | | *27320.2* | *27611.2* | | | | | |
| $Hex_{18}HexNAc_{15}Fuc_3$ | *26681.2* | 26972.2 | 27263.2 | 27554.2 | | | | | |
| $Hex_{19}HexNAc_{16}Fuc_3$ | *27046.0* | 27337.0 | 27628.0 | 27919.0 | 28210.0 | | | | |
| $Hex_{20}HexNAc_{17}Fuc_3$ | | 27702.0 | 27993.0 | 28284.0 | 28575.0 | 28866.0 | 29157.0 | | |
| $Hex_{21}HexNAc_{18}Fuc_3$ | | *28068.0* | 28358.7 | 28649.7 | 28940.7 | 29232.0 | 29523.0 | | |
| $Hex_{22}HexNAc_{19}Fuc_3$ | | *28433.4* | 28724.4 | 29014.7 | 29305.7 | 29596.7 | 29887.7 | 30179.2 | 30469.7 |
| $Hex_{23}HexNAc_{20}Fuc_3$ | | | *29089.2* | 29380.2 | 29671.3 | 29962.4 | 30253.5 | 30544.5 | |
| $Hex_{24}HexNAc_{21}Fuc_3$ | | | *29453.7* | 29745.5 | 30036.5 | 30327.5 | 30618.7 | 30909.6 | |
| $Hex_{25}HexNAc_{22}Fuc_3$ | | | *29819.7* | *30109.7* | 30401.7 | 30693.0 | 30984.0 | 31275.0 | |
| $Hex_{26}HexNAc_{23}Fuc_3$ | | | *30185.6* | *30476.6* | 30767.6 | 31058.6 | 31349.6 | 31640.6 | |
| $Hex_{27}HexNAc_{24}Fuc_3$ | | | | | *31132.9* | *31423.9* | *31714.9* | *32005.9* | |
| $Hex_{28}HexNAc_{25}Fuc_3$ | | | | | *31496.7* | *31789.2* | *32080.2* | *32371.2* | |

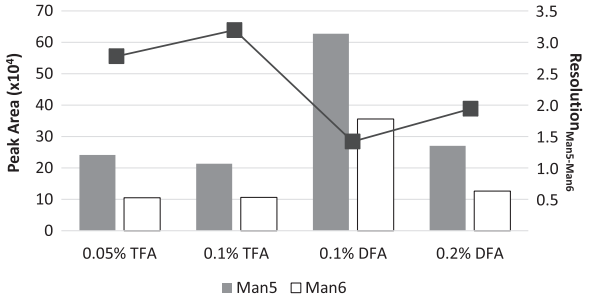


**Fig. 4.** Peak area (bars, left axis) and resolution (■, right axis) of the Man-5 and Man-6 glycoforms of RNAse B obtained during HILIC-MS using 0.05–0.1% TFA and 0.1–0.2% DFA as mobile phase additive. Further conditions, see Experimental Section.

glycoform resolution. Somewhat better efficiencies and resolution was obtained by increasing the percentage DFA to 0.2%, but detection sensitivity reduced, leading to glycoform peak areas similar to the ones obtained with 0.1% TFA (Fig. 4 and S3).

HILIC-MS of rhEPO using 0.1% DFA as additive resulted in a significantly better signal-to-noise ratio and some loss of glycoform resolution as compared to 0.1% TFA (Fig. 5A). In fact, looking at the spectrum obtained for the main peak obtained with DFA, a number of signals corresponding to coelution of several protein glycoforms can be observed. Notably, the mass spectra obtained for rhEPO with DFA showed a very different charge-state distribution as obtained using TFA, as depicted for the most abundant glycoform in Fig. 5B. With TFA, the charge-state distribution was centered around the 8 + ion, whereas with DFA the distribution shifted to higher charge states with the 12 + ion being the most intense. TFA anions are known to decrease the overall charge of gas-phase protein ions by forming adducts that do not dissociate during the ESI process [23,30]. DFA is a less strong ion-pairing agent and potential DFA-protein adducts dissociate during ESI, overall resulting in higher protonation of the protein, but also a wider charge-state distribution. The latter is not favorable from a sensitivity point of view, as the total glycoform signal is spread over a larger

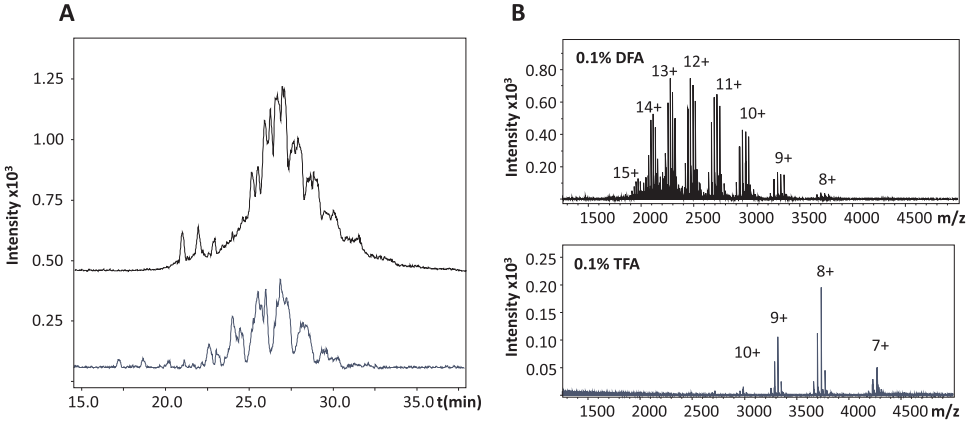


**Fig. 5.** HILIC-MS of rhEPO. (A) Base-peak chromatograms and (B) mass spectra recorded in the main peak using (bottom traces) 0.1% TFA or (top traces) 0.1% DFA as mobile phase additives. Gradient, 28–33% B in 7 min and from 33% to 40% in 28 min followed by 40% B for 10 min. Further conditions, see Experimental Section.

number of species.

Deconvoluted mass spectra were generated for the peak profile obtained with HILIC-MS employing 0.1% DFA (Fig. 5A upper trace) and the obtained masses of the glycoforms were listed. The 51 glycoform compositions as found using TFA were also assigned when using DFA (Table 2). On top of that, 21 more minor glycoforms (some with relative abundances below 1% with respect to the most abundant glycoform) were revealed (masses printed in italic in Table 2). It appears that the partial loss of glycoform resolution that is suffered when using DFA, is compensated for by a gain in detection sensitivity, overall allowing detection of more rhEPO glycoforms.

## 4. Conclusions

This paper demonstrates the usefulness of amide HILIC-MS for profiling of intact therapeutic glycoproteins. Glycoforms of rhIFN-β − 1a and rhEPO could be efficiently separated using a superficially-porous amide-bonded stationary phase in combination with rather shallow ACN-water eluent gradients containing an ion-pairing agent. Under these conditions, glycoprotein retention is dominated by the neutral sugar content of the glycans present. The achieved separation enabled characterization of a large number of proteoforms in one single HILIC-MS run. This kind of information on intact proteins is difficult to obtain with current LC-MS techniques, such as RPLC-MS. The possibility of direct injection of aqueous formulations adds even more to the practical value of HILIC-MS for the quality control of biopharmaceuticals. Sensitivity issues posed by the use of TFA as eluent additive, could (at least partially) be circumvented by applying ISCID to dissociate TFA-glycoprotein adducts or by replacing TFA for the weaker ion-pairing agent DFA. Currently we are working on further improvements of HILIC-MS sensitivity for intact proteins by testing capillary HILIC columns in combination with trap-column technologies with the aim to enhance ESI efficiency as well as increase sample volume loadability.

## Conflict of interest

The authors declare no conflict of interest

## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2018.03.015.

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