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Analysis of Carbohydrate Moieties of Recombinant and Natural Peptide Hormones by LC-MSMS

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Abstract

Glycoprotein hormones and their recombinant counterparts are abused by athletes seeking to gain a competitive edge. Erythropoietin (EPO) is used to increase oxygen capacity for endurance sports [1]. The predicament that anti-doping laboratories face is that these hormones are naturally produced in the body and the distinction against their recombinant analogues is not possible based on conventional immunoassays. However, most peptide hormones undergo post translational modification, i.e. glycosylation, and there are significant differences in the nature of the attached carbohydrates between natural and recombinant hormones. The current gel-based method for the detection of doping with EPO relies on glycosylation variation allowing the differentiation between natural and recombinant EPO. This project aimed to develop a method identifying the same glycosylation variations using mass spectrometry in urinary extracts.
A workflow was established for the detection of the glycosylation variations for recombinant EPO. The development included validation of the urinary extraction of EPO using immunoaffinity columns, optimisation of the digestion and deglycosylation of EPO, identification of suitable derivatisation methodology and development of liquid chromatography separation method for use with a multiple reaction monitoring (MRM) mass spectrometric detection of the released and labelled glycans from recombinant EPO (Figure 1).
The developed method, when fully validated, will be immediately useful for confirmation purposes for samples which are within its limit of detection. This is a major step forward for the anti-doping community's ability to use widely accepted mass spectrometry technology as a confirmation for recombinant EPO.

Introduction

Hormones undergo post translational modification wherein carbohydrates are covalently linked to asparagine residues (N-linked) or to serine/threonine residues (O-linked) within the peptide backbone [2]. Glycosylation, particularly in recombinant technology, is dependent on the species and the type of cell used, which affects the resulting isoform profile [3].

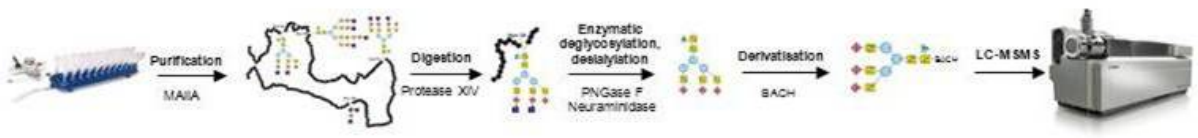

Figure 1: Workflow was established for the detection of the glycosylation variations for recombinant EPO.

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Structural analysis of carbohydrates by mass spectrometry (MS) has been conducted for many years [4]. Mass spectrometry can be used to analyse underivatised (intact) sugars, however, labelling or derivatising the reducing end of the carbohydrates can increase the sensitivity as it provides an efficient ionization mechanism [5,6]. Common chemical labels used are aryl amine reagents such as 2-aminobenzamide (2-AB) and 2-aminobenzoic acid (2-AA) [6,7]. A recent study demonstrated improved sensitivity of glycans with MS analysis through the use of a biotinylated labelling reagent (BACH) [6]. Initial labelling experiments were performed with readily available carbohydrate standards to establish conditions for compatibility and selectivity with the separation techniques utilised. Prior to labelling or derivatisation, the sugars need to be released enzymatically from the protein core with the use of enzymes such as PNGase F (N-linked) and b-elimination reagents (O-linked). Recombinant EPO and its analogues were used to develop and optimise conditions before utilising lower concentrations reflecting urinary levels. These conditions were used for analysing urine samples purified with immunoaffinity chromatography columns (IAC). The possibility of using liquid chromatography coupled with mass spectrometry for the detection of EPO would enhance the world testing program for anti-doping in sports.

Experimental

Immunopurification: Follow MAIIA anti-EPO column purification procedure [10]. Take 20 mL of urine and add 2 mL UPD, mix gently and incubate for 10 minutes. Using a water bath, heat samples to 95°C for 9 minutes then cool. Add 20 mL dilution buffer and mix. Filter using a Sartorius Minisart 45 µm filter. Condition the MAIIA anti-EPO column using a 1 mL wash buffer. Add filtered sample and allow to pass through column. Add 1 mL wash buffer and completely dry column using a centrifuge. Elute glycoprotein using 50 µL desorption buffer into an eppendorf tube containing 5 µL adjustment buffer.

Digestion: Calculate glycoprotein and ProteaseXIV 1/10 enzyme:substrate ratio. Incubate at 37°C for 72 hrs at 300 rpm using an Eppendorf Thermomixer. Stop digestion by denaturing enzyme using heat in a water bath 80°C for 5 min. Collect retentate into a 1.5 mL eppendorf tube and vacuum dry. Proceed to deglycosylation.

Denaturation and Deglycosylation: Dissolve glycoprotein in 45 µL 50 mM Tris-HCl, pH 8. Add 2.5 µL denaturation solution (2% SDS and 1 M 2-mercaptoethanol), heat at 95°C for 5 min and allow to cool. Add 2.5 µL 15% Nonidet P-40, 2 µL PNGase F and 2 µL neuraminidase, incubate using a thermomixer for 3 hours at 37°C and 350 rpm. Collect released N-glycans using a 10 kDa filter (Vivaspin 2) by centrifuge. Desalt glycans using PGC tips (Thermo) using the following protocol: Condition tips with 2 x 100 µL 80% acetonitrile/0.1% TFA, wash tip with 3 x 100 µL water, load sample, wash tip with 3 x 100 µL water, elute glycans with 3 x 100 µL 25% acetonitrile/0.05% TFA. Proceed to derivatisation.

Derivatisation: Prepare 5 mM biotinamidocaproyl hydrazide (BACH) solution, take 1 mg BACH in 530 µL 30% acetonitrile. Add 100 µL of BACH solution to dried glycan, vortex and vacuum dry. Add 30 µL of 95% methanol, incubate at 90°C for 1 hr and vacuum dry. Reconstitute and run on LCMSMS.

Analysis by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS): The samples were analysed using an Agilent 1100 series LC coupled with an ABSciex 4000QTrap LC-MSMS. Labelled glycans were separated using a TSK gel amide 80 column (100 × 1.0 mm i.d., TOSOH, Japan) at 40°C under normal phase conditions. The mobile phase consisted of 0.1% formic aicd H_2O (solvent A) and 0.1% formic acid in ACN (solvent B). The gradient separation from 0 to 1.5 min, 30% B; from 1.5 to 8 min, to 55% B; from 8 to 15 min, 55% B; from 15 to 29.5 min, to 95% B; during 1 min, 95% B; from 30.5 to 31 min, to 30% B; from 31 to 34 min, 30% B, at a flow rate of 0.05 mL/min. Data was acquired using Analyst (Version 1.5.1) software in Precursor Ion mode initially to develop methodologies and then in multiple reaction monitoring mode (MRM).

Results and Discussion

Derivatisation of glycan standards: Labelling experiments were carried out using aryl amine reagents such as 2-aminobenzamide (2-AB), 7-amino-4-methylcoumarin (AMC), 6-aminoquinoline (6-AQ) and 2-aminoacridone (2-AMAC). Other types of derivatisation reagents were also reviewed including biotin derivatives (e.g. biotinamidocaproyl hydrazide (BACH)), 1-phenyl-3-methyl-5-pyrazolone (PMP) and 9-Fluorenylmethylchloroformate (Fmoc-Cl). These labelling reagents

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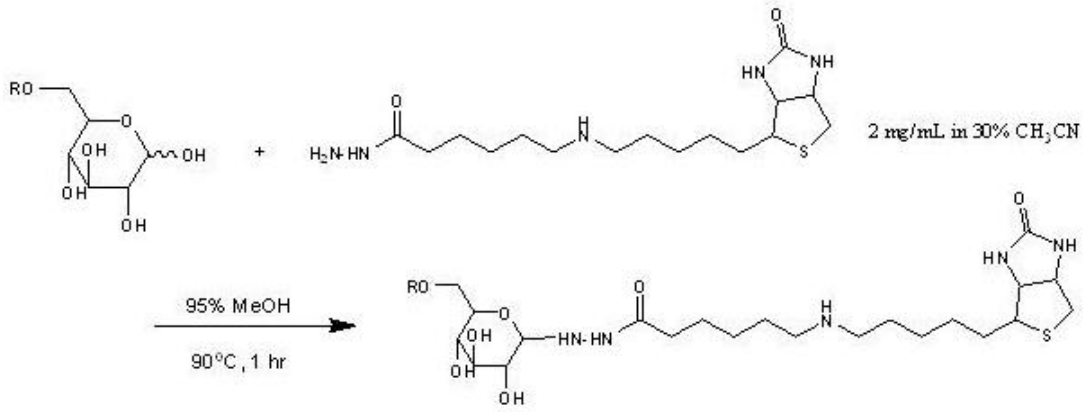
were used to derivatise commercially available glycan standards; NA2, NA2F, NA2FB, NA4 and SLN. The methods used for chemical derivatisation were based on reported techniques from current literature [4,5,6,7] while the LC-MSMS analysis conditions were developed in the laboratory and optimised for each reagent as required.

Several of the more successful reagents were chosen to continue with further experimentation, applying the technique to glycosylated proteins could change their effectiveness when compared to labelling glycan standards. 2-AB, AMC and BACH were used as labelling agents with N-glycans which had been released from recombinant EPO. Efficiency of labelling is important when dealing with low levels of glycans in biological medium. Labelling with biotinamidocaproyl hydrazide (BACH) of glycans was achieved through a non-reductive hydrazide-mediated coupling as shown in Scheme 1. Sample clean-up was not necessary as the BACH reagent was not used in excessive amounts compared to labelling by reductive amination. Overall, the BACH reagent was found to be the quickest and most effective of the labelling techniques performed as there was no purification required before analytical analysis, reducing sample preparation time.



Scheme 1: Reaction showing the non-reductive hydrazide-mediated coupling of a biotin derivative to a glycan. R = extended glycan. 1 Kapkova, P. Rapid Comm Mass Spectrom, 2009 23:2775-2784

Digestion and N-linked Deglycosylation: It was found that digestion of the protein was necessary prior to deglycosylation. Two enzymes were tested, Trypsin and Protease XIV. Trypsin hydrolyses only the C-terminal side of lysine and arginine amino acid residues and no cleavage occurs if a proline residue is on the carboxyl side of the cleavage site. Protease XIV is a pronase mixture which is nonspecific reducing the majority of proteins to mono-amino acids. A combination of digestion with Protease XIV followed by deglycosylation with PNGase F was found yield the most glycan isoforms. Experimentation with different Protease XIV digestion incubation times was explored. It was found that whilst 48 hours digestion at 38°C before deglycosylation was adequate, better results were achieved when the digestion time was increased to 72 hours. Two distinguishable glycan isoforms of Eprex® with good baseline separation were observed at 12.8 and 14.2 mins (Figure 2). Experiments were performed using PNGase F for the removal of N linked sugars on digested glycoproteins. Neuraminidase enzyme was also added in some experiments to investigate glycosidic linkages of sialic acids. These were compared to unlabelled (free) N-glycans released from recombinant EPO (Eprex®). Further experimentation with BACH labelling of both desialylated and intact sialic acids of Eprex® was explored. Clear peaks were identified after desialylation with an improvement in the peak separation and number of isoforms present when sialic acids were removed.

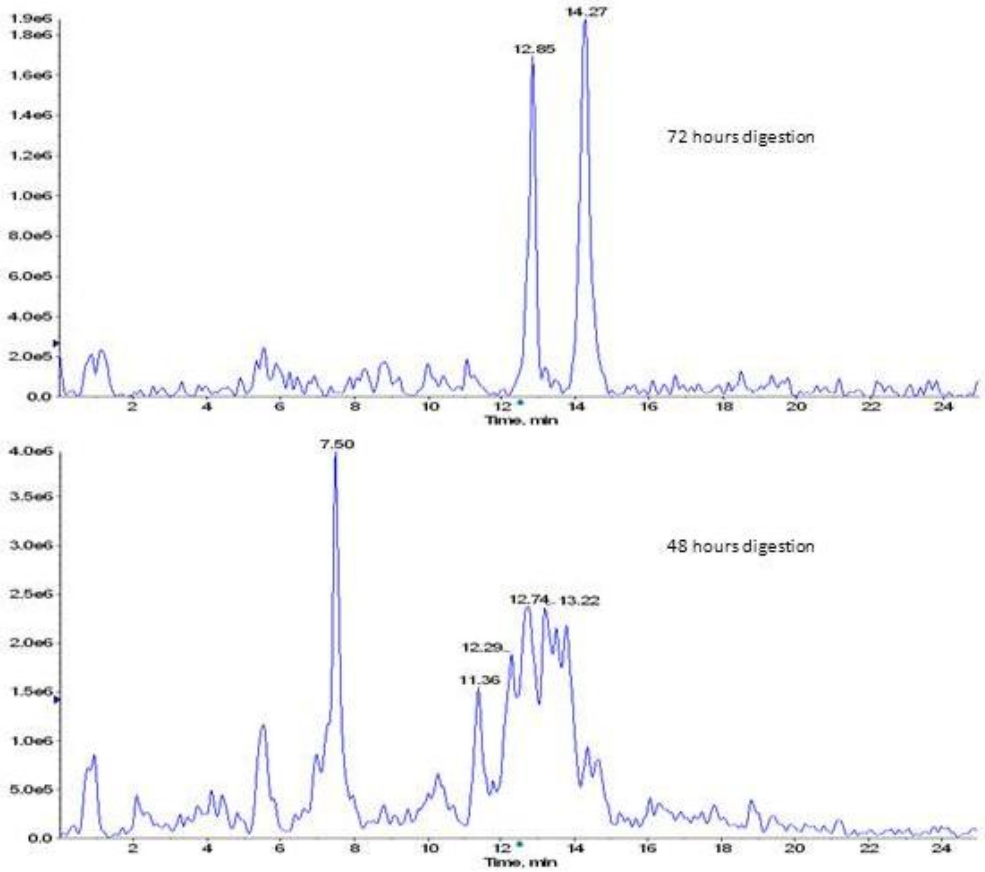


Figure 2: Chromatograms of BACH-labelled desialylated N-linked glycans from Eprex® which has been digested with Protease XIV for 72 hours (top) and 48 hours (bottom).

Optimisation of LC-MSMS: Using the sample preparation methods discussed in the previous sections, LC-MSMS conditions were optimised to create a MRM method capable of distinguishing recombinant glycans of EPO. Initially, EPI (Enhance Product Ion) scans of the dominant *m/z* of each BACH labelled desialylated glycan of Eprex® were run to ascertain the optimum precursor and product ions. Using the information acquired from the EPI analysis, MRM methods were developed to detect Eprex® glycans separately. A 10 µg standard of Eprex® was used to optimise the method and observe the most abundant glycans. Two abundant and well separated glycans were focused on for the optimisation of Eprex®: m/z 825.8 eluting at 12.7 mins and m/z 711.3 eluting at 14.2 mins. The MRM method was optimised using the EPI scans to have two glycan isoforms for Eprex® with good baseline separation eluting at 12.7 and 14.2 mins with a CE of 28 eV (Figure 3). The final optimised MRM parameters are shown in Table 1. Eprex® spiked standards were run to establish linearity across a range of concentrations from 5 IU to 200 IU spiked in 50 µL Tris buffer (Figure 4).

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Q1 mass (Da)	Q3 mass (Da)	Dwell Time (msec)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (eV)	Exit Potential (V)
825.4	663.4	250	20	10	28	11
825.4	366	250	20	10	28	11
711.3	596.2	250	20	10	28	11
711.3	549.3	250	20	10	28	11

Table 1: MRM parameters used in the final LC-MSMS method for the analysis of recombinant EPO.

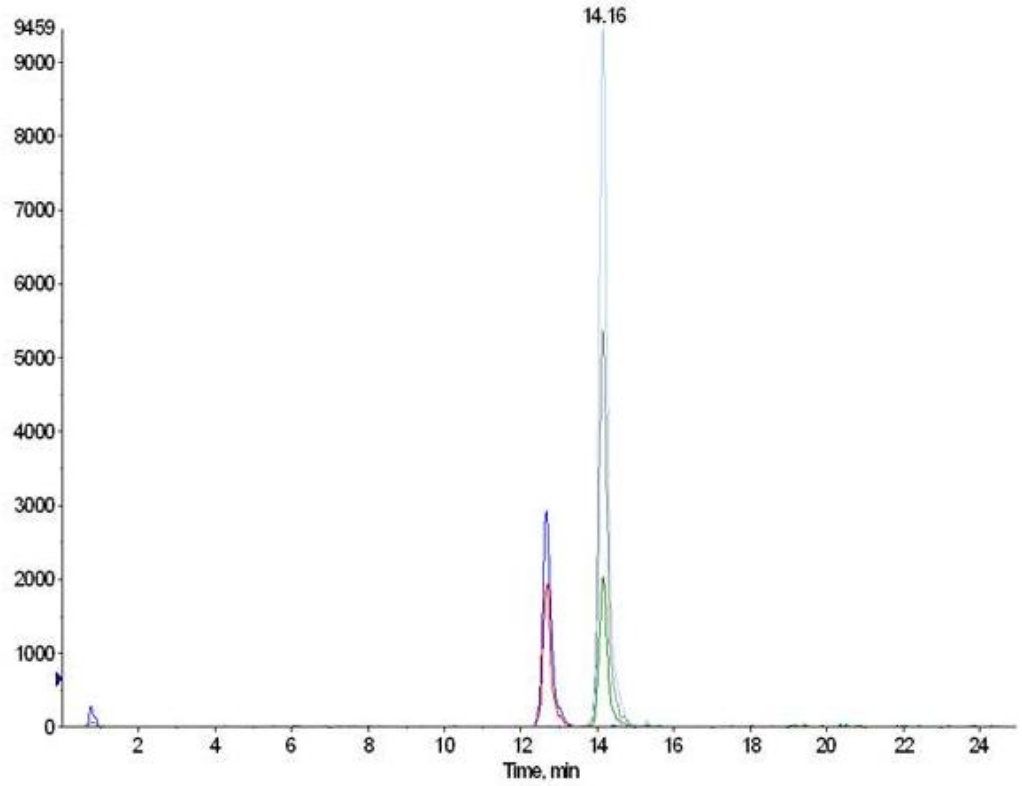

Figure 3: Chromatogram of the MRM transitions for the two abundant glycan isoforms of Eprex® at m/z 825 and 711.

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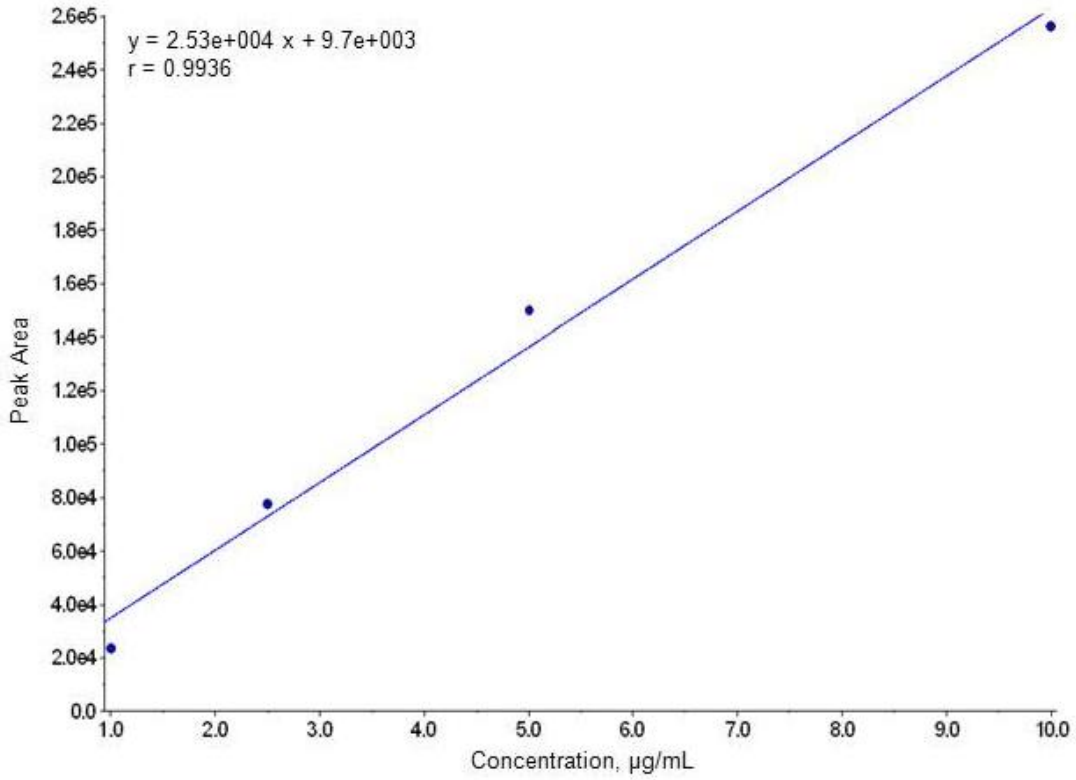

Figure 4: Calibration curve for BACH labelled N-glycans released from recombinant EPO.

Urinary Extraction of EPO:
MAIIA immunoaffinity extraction columns have been specifically developed for the isolation of EPO from urine and serum. They offer a protocol that provides purified EPO extracts suitable for deglycosylation and derivatisation prior to mass spectrometry analysis. The urine clean-up procedure has been combined with the optimised derivatisation and deglycosylation procedure in an initial trial to analyse urine spiked with Eprex®. The workflow depicted in Figure 1 is the expected procedure for analysing labelled glycans released from purified urinary EPO. However this entire process now awaits validation and its comparison with samples containing natural EPO at an appropriate level remains to be carried out.

Conclusions

In this study it was shown that BACH labelled, N-glycans of recombinant EPO could be accurately and reproducibly detected by LC-MSMS analysis. The combination of MAIIA urinary EPO purification, digestion using Protease XIV, enymatic deglycosylation with PNGase F whilst desialylating with neuraminidase, derivatisation of the N glycan through a non-reductive hydrazide-mediated coupling to BACH and finally analysis with the LC-MSMS was found to be the most effective method. This method, when validated, has the potential to be utilised as a screening or confirmation technique for the detection of recombinant EPO in athlete samples. This is a major step forward for the anti-doping community's ability to use widely accepted mass spectrometry technology as a confirmation technique.

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