High-performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAE-PAD) – An Effective Tool for Analysing Monosaccharides and Sialic Acids in Biosimilars

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Most glycoprotein therapeutics have either lost patent protection or will be losing their patent protection in the near future, accelerating the development of biosimilars. These glycoproteins exhibit complex molecular structure, most notably in their glycosylation pattern. The variations in glycosylation can affect therapeutic's efficacy and safety. Thus, it's critical to establish the biosimilarity of biosimilars compared to the original glycoprotein drug. Recent regulatory guidance on biosimilar development requires sponsor to show that a candidate product is highly similar to the originator reference product at the analytical level, including amino acid sequence, higher-order structures, glycosylation, pegylation, etc, as well as structural characteristics. This regulatory guidance for biosimilar development is driving demand for monosaccharide and sialic acid analyses, which is used for monitoring biosimilar glycosylation. This article presents how High-performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAE-PAD) can be utilised for the separation and direct quantification of these non-derivatised carbohydrates.

Introduction

Most of the top leading biotherapeutics have either lost patent protection or are expected to lose their patent protection by 2019, resulting in an increased interest to develop biosimilar therapeutics [1]. In addition to the patent cliff, the high cost of these leading biologics also led to the significant growth towards the development of these of biosimilars. According to FDA regulations, for a biosimilar to get approved, it must have a highly similar structure and exhibit no clinically meaningful differences from the reference product in terms of the safety, purity, and potency of the product [2-3]. Meeting these strict structural and clinical parameters can be very challenging due to the complex nature of these biologics and their related bioprocesses and manufacturing.

The majority of biotherapeutics are glycoproteins manufactured via a complex process, using recombinant DNA technology in live cells [4]. During glycoprotein production, an enzymatic post-translational modification process attaches glycans, via a process known as glycosylation, to the proteins affecting the final efficacy and safety of the product [5]. The glycosylation profile depends on many manufacturing factors such as media, cell line, culture conditions and bioreactors [5]. A key step to proving biosimilarity is to ensure proper N-glycosylation such as glycoprotein sialylation using the appropriate analytical technology. HPAE-PAD provides biosimilar manufacturers a highly effective method for the separation and direct quantification of these non-derivatised carbohydrates.

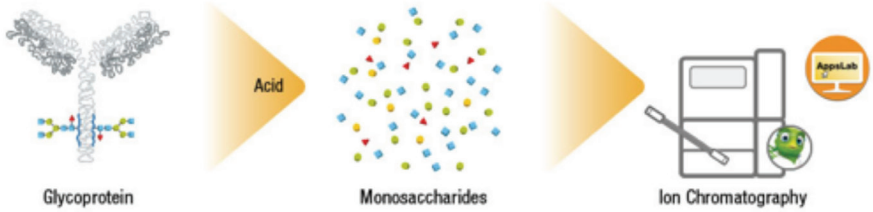

Figure 1. Glycoprotein Monosaccharide Analysis by HPAE-PAD.

What is HPAE-PAD?

HPAE chromatography at high pH coupled with PAD is one of the most employed techniques for carbohydrate compositional analyses for either routine monitoring or research applications. The compatibility of electrochemical detection with gradient elution allows mixtures of simple sugars and oligo- and polysaccharides to be separated with high resolution in a single run. This technique has great impact on the analysis of diverse carbohydrates ranging from mono-, di-, and polysaccharides. Direct detection analysis of carbohydrates is quite challenging due to their inherent highly polar structure and the absence of a suitable chromophore. HPAE takes advantage of the weakly acidic nature of carbohydrates to give highly selective separations at a high pH using a strong anion-exchange stationary phase [6]. Coupled with PAD, it permits direct detection and quantification of non-derivatised carbohydrates at high femtomolar concentration levels with minimal sample preparation and cleanup [6].

Fundamentals of HPAE-PAD

HPAE chromatography separates anions under high pH conditions. Carbohydrates typically have pK_as in the range of 12–13. Once the pH rises above the pK_a of the

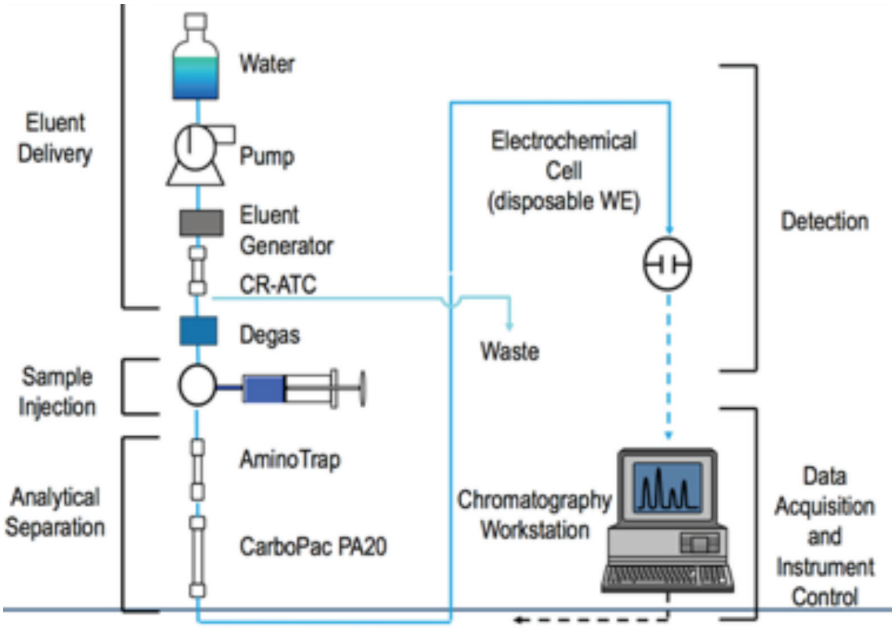

Figure 2. HPAE-PAD Glycoprotein Monosaccharide System

carbohydrate, these carbohydrates ionise (more specifically, become oxyanions) and are then separated. These separations require hydroxide-based eluents. For hybrid or complex carbohydrates, separations are accelerated and improved by using sodium acetate gradients in sodium hydroxide. The analytes are separated using one of an extensive Dionex CarboPac column portfolio developed specifically for carbohydrates ranging from mono-, di-, oligo-, and polysaccharides. These highly cross-linked, ethylvinyl benzene-divinyl benzene pellicular resins have broad pH stability ranging from 0 to 14, allowing separations at high pH conditions. Silica based stationary phases are not stable in high pH mobile phases, limiting their use in carbohydrate separations using an ion exchange mechanism [7].

Once separated, these non-derivatised carbohydrates are detected on a gold working electrode (WE) surface by PAD, which is a direct detection technique. Alternative direct detection techniques available for LC of carbohydrates are short wavelength ultra-violet light (UV) and refractive index (RI), both of which lack the sensitivity required for analysing mono- and oligosaccharides from glycoproteins [7] [6]. Additionally, the detection of these non-derivatised carbohydrates using UV is limited to the choice of the solvent being used. As most of the solvents and carbohydrates absorb quite strongly below 200 nm. PAD easily addresses the sensitivity issue, as it is able to routinely detect low picomolar amounts of carbohydrates. This method applies a series of potentials (a waveform) applied two times per second (2 Hz) to a gold working electrode, at high pH, resulting in the oxidation of analytes bound to the working electrode surface. PAD only detects compounds that contain functional groups that can get oxidised at the detection voltage. Detection is sensitive and highly selective for electroactive species, as many potentially interfering species cannot be oxidised or reduced, and so are not detected.

Glycoprotein Monosaccharide Analysis

A comprehensive monosaccharide profiling is required for biosimilars to meet FDA regulations. This comprehensive monosaccharide analysis helps in the identification and quantification of the different types of glycosylation occurring in these proteins. In the biosimilar development process, it is critical to analyse monosaccharides in earlier stages to address any issues related to their manufacturing process. This information will prevent the development of an inappropriate products that would not meet the criteria stipulated by the FDA for biosimilars. For example, the differences between O- and N-linked glycans is a critical piece of information, which can easily be determined using HPAE-PAD. This will help manufacturers evaluate their bioprocess which in turn highlights whether the biotherapeutic's structural integrity and expected effectiveness can be achieved.

Sample Preparation: To prepare glycoprotein samples ahead of injection on the HPAE-PAD system for monosaccharide analysis - a sample with PNGase F were first hydrolysed with slightly varied conditions for neutral sugar or amino sugar analyses (Figure 1). The acid-hydrolysed samples were dried in a SpeedVac concentrator (Thermo Scientific) equipped with an acid trap and reconstituted in a small volume of deionised water [7].

Experimental Parameters: Columns: 3×150 mm, Dionex CarboPac PA20 (Thermo Scientific, Sunnyvale, USA) and 3×30 mm, AminoTrap (Dionex); eluent: 10 min 100 mM Potassium Hydroxide (KOH) wash followed by 10 min of equilibration at 10 mM KOH; eluent source: Dionex EGC III and CR-ATC; flow rate: 0.5 mL/min; injection volume: 5 µL; temperature: 30°C. Samples were acid hydrolysates of specified proteins. Peak results are shown in pmol.

The sample is then injected into the HPAE-PAD system and the monosaccharides are separated using a Dionex CarboPac PA20 column (Thermo Scientific, Sunnyvale, USA) (Figure 2). The Dionex CarboPac PA20 delivers excellent monosaccharide resolution while minimising mobile phase consumption and waste generation. The eluent generator eliminates error associated with hydroxide mobile phase preparation (e.g. variable carbonate contamination and therefore variable retention times), and shortens analysis time. Inclusion of a Dionex AminoTrap column (Thermo Scientific, Sunnyvale, USA) delays the elution of amino acids and small peptides from the glycoprotein acid hydrolysis so that they do not interfere with monosaccharide quantification. After separation, the monosaccharides are detected on a gold working electrode.

Figure 3 shows the sensitivity of monosaccharide analysis that can be achieved using HPAE-PAD. The amounts of galactosamine (GalN) and glucosamine (GlcN) have been determined as an indicator of O- and N-glycosylation, respectively. Figure 3 demonstrates the separation of monosaccharides present in Protein Serum Album (PSA) and PSA when treated with PNGase F. The separation of PSA acid hydrolysates was achieved on a Dionex CarboPac PA20 column. The identification of the monosaccharides present in both the treated and untreated PSA were compared with the monosaccharide standards, highlighting the sensitivity of the HPAE-PAD system. The system successfully identifies the loss of galactosamine and glucosamine after PSA treatment with PNGase. In both cases, glucosamine and galactosamine were present in roughly the same molar ratio of 0.07. This suggests that for this PSA sample, the galactosamine was not a result of O-glycosylation but is associated with the PSA N-glycans, or that there was a contaminating protein with O-glycosylation that is not removed during the sample preparation to separate protein from released N-glycans.

Sialic Acid Analysis

Sialic acids are critical to therapeutic glycoprotein efficacy, bioavailability,

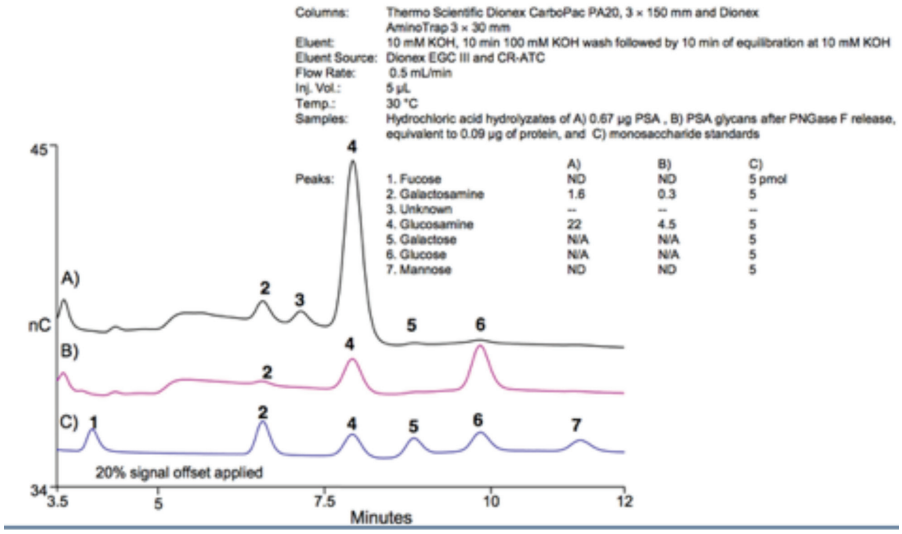

Figure 3. Sensitive Glycoprotein Monosaccharide Analysis

function, stability, and metabolism. When present, they occupy terminal positions in glycosylated proteins, providing charged points of interaction essential in many biological pathways. Any unexpected sialylation in biosimilars can cause immunogenicity, so it is critical to monitor total glycoprotein sialylation, and the identification of the sialic acids when changing process and or conditions.

Although over 50 natural sialic acids have been identified, two forms are commonly determined in therapeutic glycoprotein products: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc). Due to the potential immunogenicity of Neu5Gc, it is considered undesirable in therapeutic proteins and must be tested for [7]. HPAE-PAD offers a sensitive detection technique to determine and quantify the total sialic acid, Neu5Ac and Neu5Gc, content as well as the determination of their (Neu5Ac and Neu5Gc) relative amounts in a given glycoprotein sample.

Sample Preparation: Neu5Ac and Neu5Gc, are negatively charged at pH 7 and acid-labile so require acetate in the eluent and weak acid conditions for hydrolysis. Before injection into the HPAE–PAD system, the samples were subjected to acid hydrolysis or treated with neuraminidase to release the sialic acids, dried to remove the acid, and reconstituted in deionised water.

Experimental Parameters: Column: 3 x 30 mm, CarboPac PA20 Fast Sialic Acid (Thermo Scientific, Sunnyvale, USA); eluent: 70–300 mM acetate in 100 mM Sodium Hydroxide (NaOH) from 0–2.5 min, 300 mM acetate in 100 mM NaOH from 2.5–2.9 min, 300–70 mM acetate from 2.9–3.0 min, 1.5 min of equilibration at 70 mM acetate in 100 mM NaOH; flow rate: 0.5 mL/min; injection volume: 4.5µL (full loop injection); temperature: 30°C; detection: PAD, Au on PTFE, 2 mil gasket. Peak results are shown in pmol.

Separations are performed using either a Dionex CarboPac PA20 column, or a Dionex Fast Sialic Acid CarboPac PA20 column (Thermo Scientific, Sunnyvale, USA) designed for sensitive high-throughput analyses. For example, Figure 4 shows the quantitative determination of Neu5Ac and Neu5Gc using the Fast Sialic Acid CarboPac PA20 column in the HPAE-PAD system for human alpha-acid glycoprotein, fetuin, and sheep alpha-acid glycoprotein. This information is critical in protein expression experiments for clonal selection for cell line development for therapeutic glycoproteins as well as optimising and monitoring protein production methods.

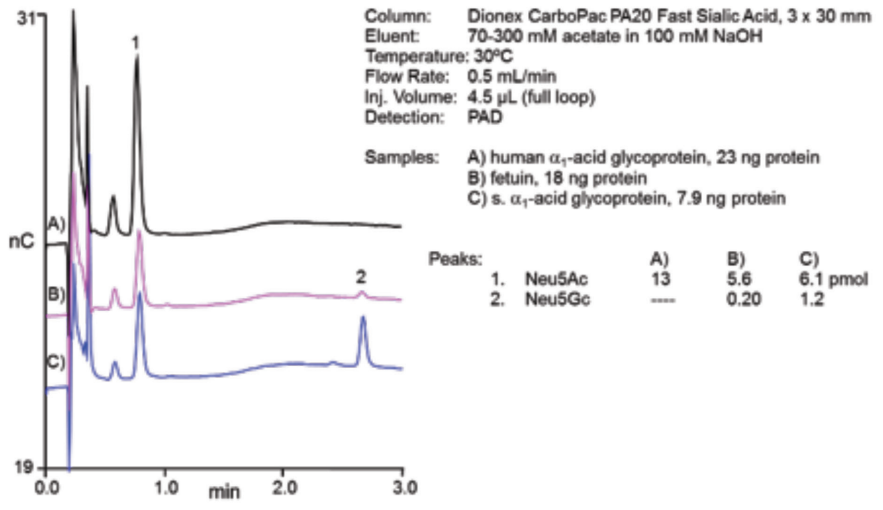

Figure 4: Fast separation of glycoprotein acid hydrolysates using HPAE–PAD

Conclusion

Biosimilars are strong candidates for leveraging themselves as alternative drugs for intended use at a much-reduced healthcare cost. However, FDA regulations must first be satisfied. Comprehensive glycosylation profiling, including sialic acid compositional analysis, is required to determine the efficacy and safety of biosimilars. HPAE-PAD is the most direct, sensitive, and selective method for monosaccharide, sialic acid, and other carbohydrate analyses.

References

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