PharmaFluidics
The micro-Chip Chromatography Company

Application Note

Detailed glycosylation analysis of therapeutic enzymes using µPAC™ capLC-MS and all-ion fragmentation

Abstract

The use of micro Pillar Array Columns (µPAC™) for characterizing glycosylation of therapeutic enzymes is presented. Recombinant human acid α-glucosidase (hGAA) was digested and resulting peptides were separated on a 50 cm µPAC™ capLC C18 column operated at low µL/min flow rate. Glycopeptide peaks were then selectively detected and identified by Orbitrap mass spectrometry (MS) operated in full MS/all-ion fragmentation (AIF) mode.

MARKET

Pharma

Biotech

KEY WORDS

µPAC™ capLC,

mass spectrometry (MS),

all-ion fragmentation,

peptide mapping,

therapeutic enzyme.

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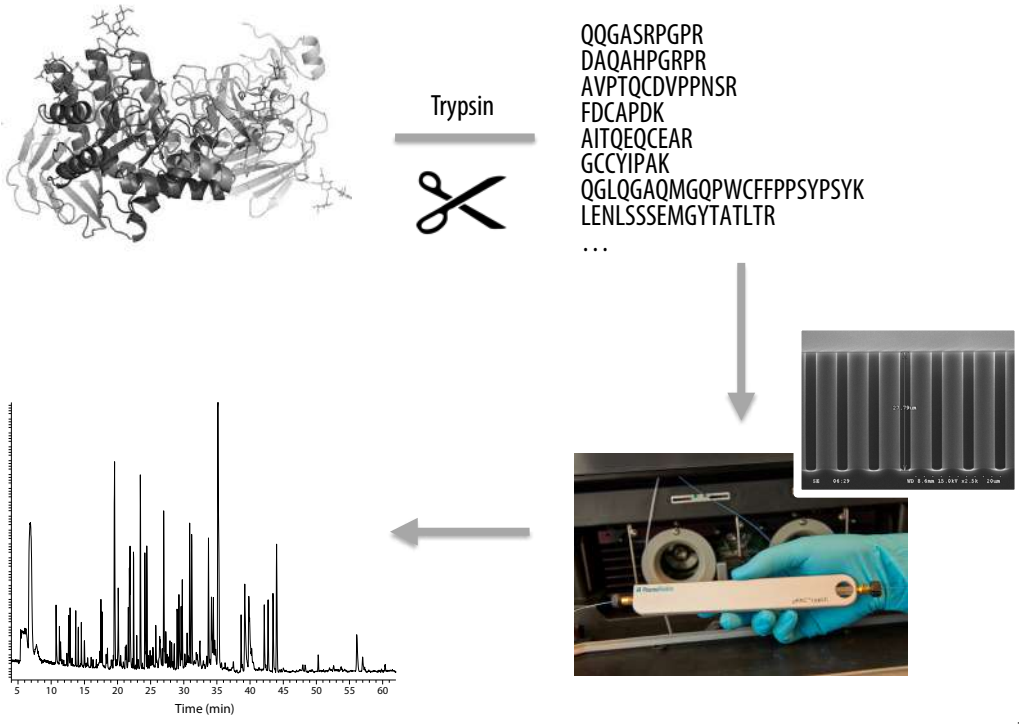

Introduction

Human acid α-glucosidase (hGAA) catalyzes the hydrolysis of glycogen to glucose in the lysosomes of the cell. There are around 50,000 people worldwide which have a deficiency of this enzyme leading to glycogen accumulation in the lysosomes, a rare and fatal disorder known as Pompe disease [1-4]. Pompe patients typically receive an enzyme replacement therapy with recombinant human acid α-glucosidase (rhGAA) commercially known as Myozyme. Recombinant hGAA is a heavily N-glycosylated protein with a MW of 110 kDa expressed in Chinese Hamster Ovary (CHO) cells. The therapeutic enzyme contains 7 N-glycosylation sites which are occupied with complex and high mannose glycans [2-4]. The former complex glycans are

predominantly sialylated and to a lesser extent acetylated, the latter glycans contain an unusual mannose-6-phosphate structure considered a critical quality attribute as it is responsible for targeting the enzyme to the lysosomal compartment of the cell where it needs to be catalytically active to break down glycogen.

To study N-glycosylation of therapeutic enzymes, glycans are commonly liberated from the protein backbone using PNGase F, fluorescently labeled and separated using hydrophilic interaction liquid chromatography (HILIC) [5-6]. While this methodology provides a wealth of information, site specific data is lost, i.e. which glycans are conjugated to which asparagine residue and to which extent. To obtain the latter, peptide mapping is required. The main challenge when performing peptide mapping is the increase of complexity, as the digestion of this therapeutic enzyme will result in hundreds of peptides with varying physicochemical properties present in a wide dynamic concentration range. Micro Pillar Array Columns (µPAC™s) are perfectly suited to tackle this complexity [7-9]. The inherent high permeability and low 'on-column' dispersion obtained by the perfect order of the separation bed makes µPAC™ based chromatography unique in its kind and offers several advantages compared to conventional column technologies (packed beds and monoliths). The peak dispersion originating from heterogeneous flow paths in the separation bed is eliminated (no A-term contributions) and therefore components remain much more concentrated (sharp peaks) during separation. The freestanding nature of the pillars also leads to much lower backpressure allowing the use of long columns. These properties result in excellent chromatographic performance with high-resolution and high sensitivity.

This application note demonstrates how micro Pillar Array Columns (µPAC™) operated at capillary flow rates in combination with Orbitrap mass spectrometry (MS) operated in all-ion fragmentation (AIF) mode come in as a very powerful tool to study glycosylation of rhGAA.

Materials and methods

Materials

Water, acetonitrile and formic acid were purchased from Biosolve (Valkenswaard, The Netherlands). Dithiothreitol (DTT) and 2-iodoacetamide (IAA) were from Sigma-Aldrich (St. Louis, MO, USA). Tris-HCl pH 7.5 was purchased as a 1M solution from Thermo Fisher Scientific (Waltham, MA, USA). Porcine sequencing grade modified trypsin was acquired from Promega (Madison, WI, USA) and Rapigest from Waters (Milford, MA, USA). Myozyme was obtained from Sanofi-Genzyme (Cambridge, MA, USA).

Sample preparation

To a volume corresponding to 25 µg of therapeutic enzyme, 26.4 µL of 0.1% Rapigest in 100 mM Tris-HCl pH 7.5 was added followed by the addition of 100 mM Tris-HCl pH 7.5 to a final volume of 46.3 µL. The sample was subsequently reduced at 60°C for 30 min by the addition of 5 mM DTT (1.2 µL of 200 mM DTT in 100 mM Tris-HCl) and alkylated at 37°C for 1 h by adding 10 mM IAA (2.5 µL of 200 mM IAA in 100 mM Tris-HCl). Digestion proceeded for 16 h at 37°C using trypsin as protease added at an enzyme to substrate ratio of 1/25 (w/w). Lyophilized trypsin (20 µg) dissolved in 100 mM Tris-HCl (50 µL) was added in a volume of 2.5 µL resulting in a final sample volume of 52.5 µL.

LC-MS

An Ultimate 3000 RSLCnano system (Thermo Fisher Scientific) was used for LC-MS measurements. Tryptic digest was analyzed on a 50 cm C18 µPAC™ capLC column (PharmaFluidics, Zwijnaarde, Belgium) maintained at 50°C. Elution was carried out with a linear gradient of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile/water (80/10 v:v), from 2% B to 70% B in 60 min with a flow rate of 2 µL/min. The µL PickUp program allowed the injection of 1 µL sample in between two plugs of mobile phase A. Loop size was 1 µL and autosampler was kept at 10°C.

High-resolution accurate mass measurements were performed on a Q Exactive plus Quadrupole-Orbitrap™ mass spectrometer (Thermo Fisher Scientific) equipped with a Nanospray Flex™ source (Thermo Fisher Scientific). The µPAC™ column was connected via a 20 µm ID/360 µm OD fused silica capillary to a PicoTip emitter (10 µm tip ID – New Objective, Woburn, MA, USA) via a µPAC™ Flex iON Connect (PharmaFluidics). Spray voltage was set at +2.1 kV, the capillary temperature was 250 °C and a S-lens RF level of 50 was used. The Q Exactive plus was operated in either full MS-ddMS2 (TopN) or full MS-AIF mode

where a MS1 survey scan is followed by a data-dependent MS/MS or AIF scan, respectively. MS1 spectra were collected from 200 to 2000 m/z at a resolution of 70,000 at m/z 200. AGC parameters included 3e6 target value and maximum injection time of 50 ms.

For ddMS2 (TopN), the 10 most abundant multiply charged precursors were selected for fragmentation by HCD (28% NCE). MS/MS spectra were acquired at a resolution of 17,500 at m/z 200 and MS2 AGC parameters included target of 1e5 and maximum injection time of 200 ms. Precursor isolation window was set to 1.2 m/z and dynamic exclusion was set to 30 s. For AIF, all precursor ions were transferred to the HCD cell for fragmentation (28% NCE). AIF spectra were collected from 80 to 1200 m/z at a resolution of 70,000. AGC parameters included 3e6 target value and maximum injection time of 50 ms.
LC-MS data were acquired in Xcalibur (Thermo Fisher Scientific) and data analysis was performed in Freestyle and BioPharma Finder (Thermo Fisher Scientific).

Results and discussion

In this study, peptide mapping was performed on a µPAC™ capLC column with a depth of 28 µm, a width of 1 mm and a length of 50 cm (**Figure 1**). These dimensions are roughly 3 times wider and 1.5 times deeper than the µPAC™ columns used earlier for peptide mapping of protein biopharmaceuticals [10]. This µPAC™ capLC column targets the 1-15 µL/min flow range typical for capillary LC and represents a compromise between robustness and sensitivity while maintaining the chromatographic performance characteristic for micro pillar-based columns. The extra robustness offered is of great importance in the highly regulated (bio)pharmaceutical industry.

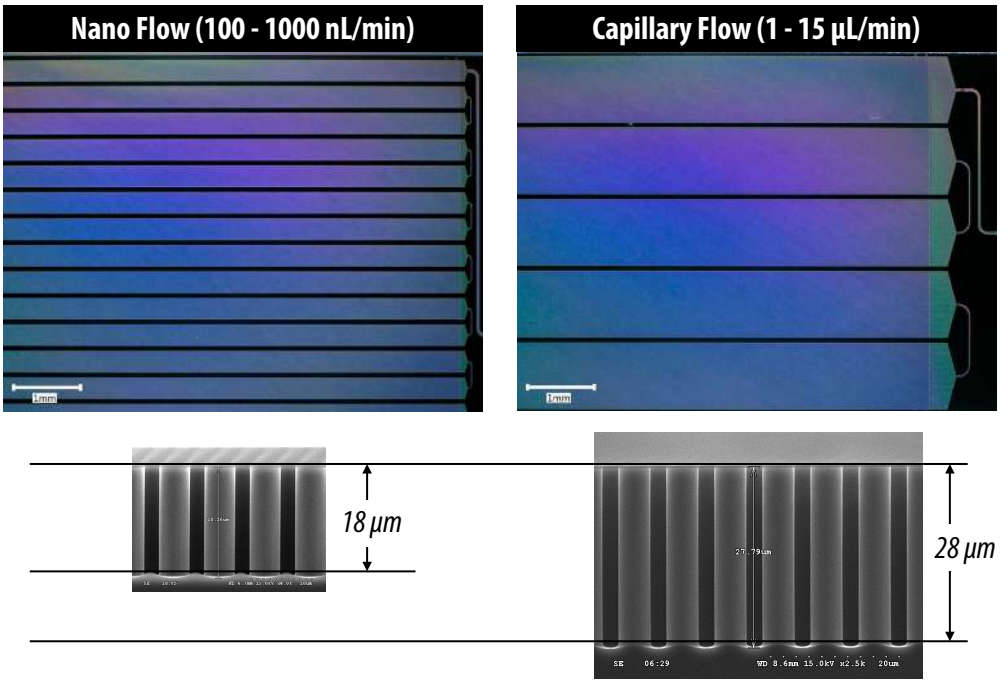

Figure 1. µPAC™ column with 315 µm wide and 18 µm deep separation channel and 5 µm diameter cylindrical pillars typically operated in the nano flow range (left). µPAC™ capLC column used in this study with 1 mm wide and 28 µm deep separation channel and 5 µm diameter cylindrical pillars typically operated in the capillary flow range (right).

Figure 2 shows the µPAC™-MS chromatograms of the rhGAA digest acquired in data dependent mode in which a full MS survey scan is followed by a set of dependent MS/MS scans. Excellent chromatographic performance is obtained using formic acid as mobile phase additive resulting in a sequence coverage of over 95%, with the majority of peptides MS/MS confirmed.

Upon higher-energy collisional dissociation (HCD), glycosylated peptides give rise to specific fragments originating from the glycan part. These sugar oxonium ions can be used to selectively recognize glycosylated peptides in the µPAC™-MS chromatogram. In order to achieve this, one can operate the mass spectrometer in the all-ion fragmentation (AIF) mode in which all peptides are transferred into the HCD cell where they are fragmented. By alternating AIF with full MS, precursors giving rise to sugar oxonium ions can be revealed and a detailed study of glycosylation sites achieved. **Figure 3** and **Figure 4** (zoom) show

the full MS chromatogram (A) and the AIF chromatograms extracting the sugar oxonium ions at m/z 204.0867 (B), 274.0921 (C), 316.1027 (D), 358.1133 (E) and 243.0264 (F) (**Table 1**).

The ion at m/z 204.0867 corresponding to N-acetylglucosamine is shared by all N-glycans and can be used as a general marker for N-glycosylation. Consequently, **Figure 3B (Figure 4B)** reveals all glycosylated peptides. Different regions are observed in the chromatogram which in fact correspond to the 7 different glycosylation sites (**Table 2**). Note that glycosylation site 390 is spread over two regions (1 and 1') because of the partial cyclization of the N-terminal amino acid glutamine (formation of pyroglutamate). The different peaks within a region correspond to different glycans at a given glycosylation site. When extracting the oxonium ions at m/z 243.0264, 274.0921, 316.1027 and 358.1133 out of the AIF data (**Figure 3C-F – Figure 4C-F**), one can specifically visualize peptides decorated with, respectively, phosphorylated, sialylated, single acetylated and double acetylated N-glycans. The data reveals that two sites are occupied with phosphorylated glycans while all sites are decorated with sialylated N-glycans (**Table 2**). A remarkable separation is achieved based on site heterogeneity on the µPAC™ column. **Figure 5** shows the extracted ion chromatograms of selected glycopeptides associated with the glycosylation site at position 470 with consensus sequence AsnGluThr (NET). Glycopeptides elute in the following order: neutral < mono-sialylated < mono-phosphorylated < di-sialylated < di-phosphorylated. Remarkable, is the isomeric separation of a mono-sialylated bi-antennary glycopeptide which can be explained by the sialylation of the 3- or 6-antenna (**Figure 5C**). In addition, and as shown in **Figure 6**, acetylation of neuraminic acid increases retention (**Figure 6A-E**), and the more monosaccharide building blocks, the less retention (**Figure 6F-H**). The various isomeric peaks observed when extracting the acetylated glycopeptides (**Figure 6B-E**) can be explained by acetylation of different hydroxyl functions (position 4, 7, 8, 9) of N-acetyl-neuraminic acid.

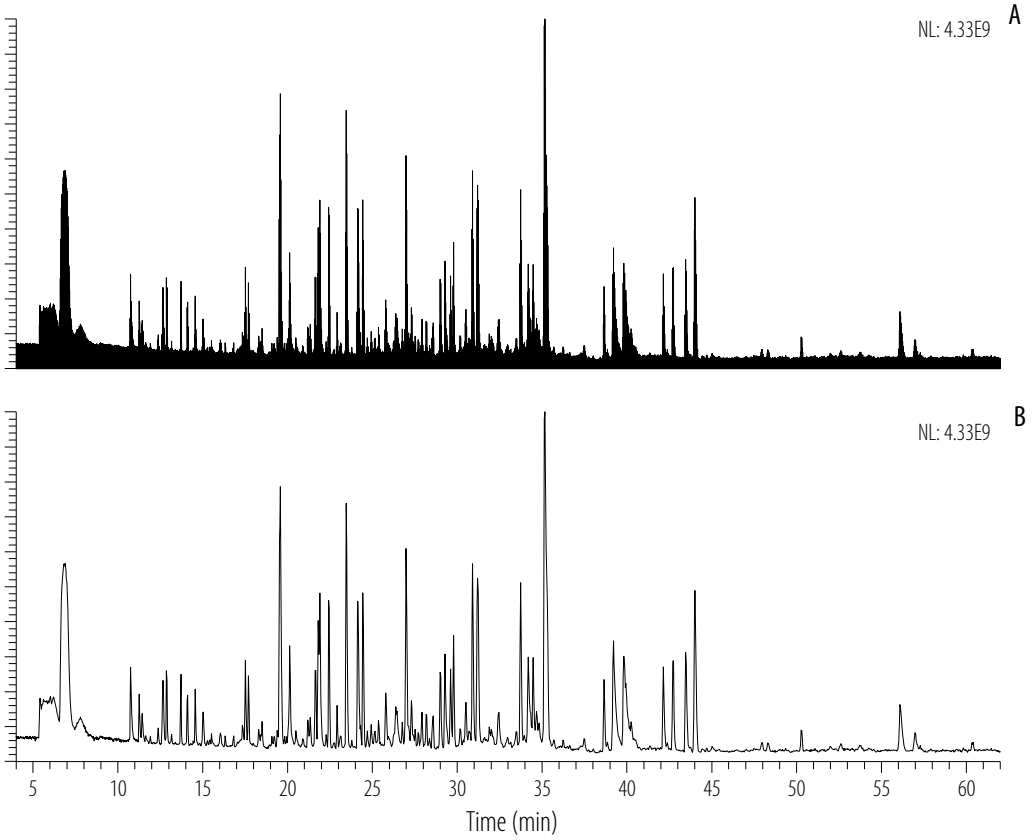

Figure 2. µPAC™-MS chromatograms of rhGAA digest with (A) TIC of full MS and MS/MS data and (B) TIC of full MS data better reflecting chromatographic performance.

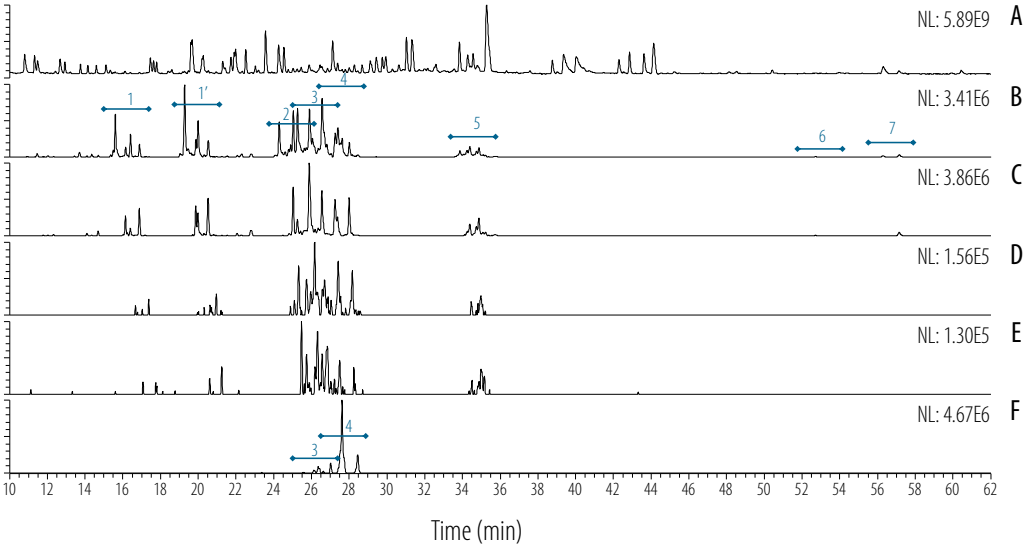

Figure 3. Full MS chromatogram (A) and the AIF chromatograms extracting the sugar oxonium ions at m/z 204.0867 for N-acetylglucosamine - GlcNAc (B), m/z 274.0921 for N-acetyl-neuraminic acid - NeuAc (C), m/z 316.1027 for mono-acetylated NeuAc (D), m/z 358.1133 for di-acetylated NeuAc (E) and m/z 243.0264 for mannose-6-phosphate – Man6P(F). Ions extracted at 5 ppm mass accuracy. Regions 1-7 correspond to specific glycosylation sites (see Table 2).

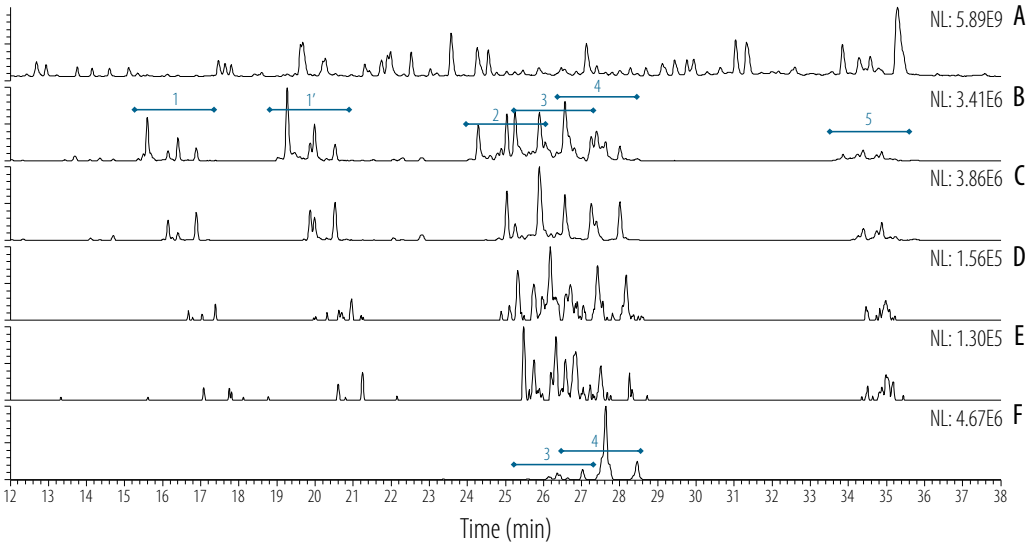

Figure 4. Zoom of **Figure 3**.

Table 1. Abbreviations and symbols used.

Monosaccharide	Abbreviation	Symbol	Sugar oxonium ion (m/z)
N-acetylglucosamine	GlcNAc	■	204.0867
Mannose	Man	○	NA
Mannose-6-phosphate	Man6P	○– P	243.0264
Galactose	Gal	○	NA
N-acetylneuraminic acid	NeuAc	◇	274.0921
O-acetylated N-acetylneuraminic acid	NeuAcOAc	◇– OAc	316.1027 (mono-acetyl) & 358.1133 (di-acetyl)

Table 2. Glycosylated peptides observed in rhGAA digest and corresponding region in chromatograms visualized in **Figure 3** and **Figure 4**.

Peptide	Glycosylation site	Predominant glycans	Region in chromatogram
QVVE**N**MTR	N390	Complex / Sialylated	1
pyroE VVE**N**MTR*	N390	Complex / Sialylated	1′
NNTIVNELVR	N882	Complex / Sialylated	2
LE**N**LSSSEMGYTATLTR	N140	Complex / Sialylated High mannose / Phosphorylated	3
GVFIT**N**ETGQPLIGK	N470	Complex / Sialylated High mannose / Phosphorylated	4
VTVLGVATAPQQVLSNGVPVS **N**FTYSPDTK	N925	Complex / Sialylated	5
YAGHWTGDVWSSWEQLASSV PEILQFNLLGVPLVGADVCGFLG **N**TSEELCVR	N652	Complex / Sialylated	6
VLL**N**TTVAPLFFADQFLQLSTSL PSQYITGLAEHLSPLMLSTSWTR	N233	High mannose	7

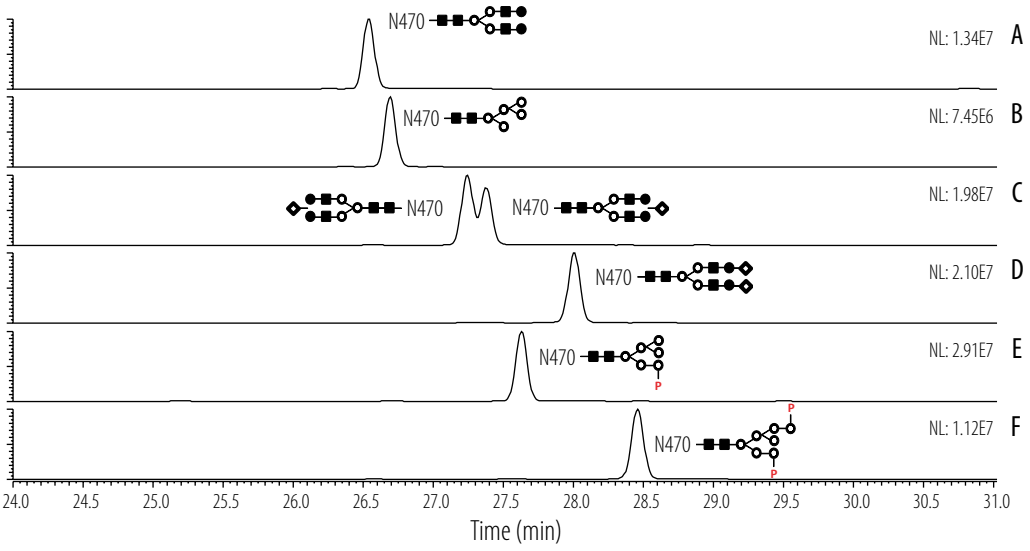

Figure 5. Extracted ion chromatograms of selected glycopeptides associated with the glycosylation site at position 470 (GVFITNETGQPLIGK) and corresponding to different glycan classes, i.e. bi-antennary complex (A), high mannose neutral (B), mono-sialylated bi-antennary (C), di-sialylated bi-antennary (D), mono-phosphorylated high mannose (E) and di-phosphorylated high mannose (F). Ions were extracted at 5 ppm mass accuracy.

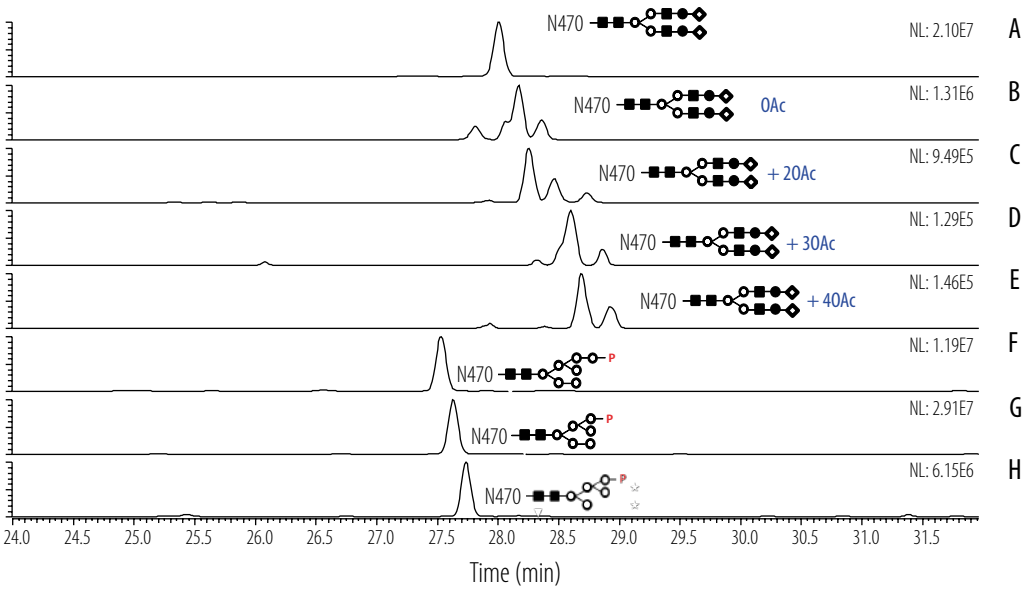

Figure 6. Extracted ion chromatograms of selected glycopeptides associated with the glycosylation site at position 470 (GVFIT**N**ETGQPLIGK) and differing in acetylation degree (A-E) and mannosylation degree (F-H). Ions were extracted at 5 ppm mass accuracy.

Conclusion

Tryptic digests of therapeutic enzymes can be more complex than antibodies due to the high numbers of glycosylation sites and their heterogenic glycan structures. The detailed study of the therapeutic glycosylated enzyme rhGAA using the µPAC™ capLC column operated at low µL/min flow rate in combination with Orbitrap MS demonstrates the high separation capacity of the µPAC™ technology for complex mixtures. In particular the isomeric separations of several sialylated and acetylated glycopeptides can be achieved, making the µPAC™ capLC column very suitable for microflow (LC-MS) separation of biotherapeutics. The resolving power offered by the µPAC™ column is primordial for an in-depth characterization and using all-ion fragmentation, glycosylated peptides can facilitate data interpretation.

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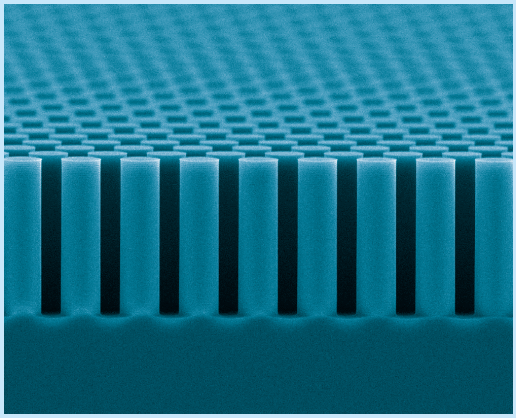
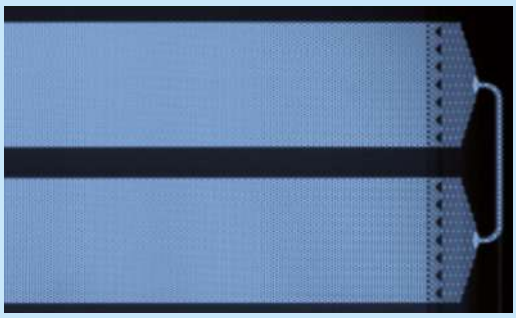
µPAC™ driven separations – Better by Design

Conventionally LC columns are fabricated by stacking (packed beds) or depositing (monoliths) material into a capillary. PharmaFluidics' µPAC™ technology (micro Pillar Array Column) is unique in its kind as it is built upon the precise micromachining of designed chromatographic separation beds into silicon. This approach brings along three crucial and unique characteristics:





Perfect Order.

µPAC™ beds are designed with a high degree of order, eliminating heterogeneous flow paths otherwise present in conventional columns (so called Eddy dispersion). Flow through µPAC™ columns adds very little dispersion to the overall separation. As a result, peaks remain sharper and sensitivity is increased.

High Permeability.

µPAC™s operate at moderate pressures, typically lower than 300 bar. Separation channels with exceptional length (50 cm to 200 cm) are therefore possible. These are folded onto a small footprint by a interconnecting concatenating bed segments.

Solid Backbone.

The micromachined backbone of the separation bed forms a rigid structure that is not influenced by pressure. There are no obstructions by touching surfaces, and there is no risk for perturbations by pressure fluctuations.

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