Application Note

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Characterization of the Anticoagulant Nadroparin by 2D-LC With High-Resolution MS

Using the Agilent 1290 Infinity II Bio 2D-LC and the Agilent 6546 LC/Q-TOF for top-down analysis of low molecular weight heparins

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Abstract

Low molecular weight heparins (LMWHs) are highly complex and heterogeneous biomolecules, which are frequently used as anticoagulant drugs. Detection of LMWH contamination is essential to ensure drug safety. Due to different possibilities of modification and degrees of polymerization (DPs) in LMWHs, sophisticated analytical techniques are needed to characterize their structural composition. This application note describes the characterization of the LMWH nadroparin using the Agilent 1290 Infinity II Bio 2D-LC system combined with the Agilent 6546 LC/Q-TOF. In the first chromatographic dimension, different DPs were resolved by size exclusion chromatography (SEC) using two Agilent AdvanceBio SEC columns. In the second chromatographic dimension, separation of different oligosaccharide compositions with the same DP was performed by ion pairing chromatography using an Agilent ZORBAX Extend C18 column. The 6546 LC/Q-TOF enabled resolution of the isotopic pattern of several different oligosaccharides occurring in nadroparin. Twenty-eight different compositions were identified highlighting the structural diversity and need for effective assays for assurance of drug quality.

Introduction

Heparin is one of the oldest biological medicines used for prevention and treatment of thrombosis. LMWHs were introduced in the 1990s as anticoagulant drugs derived from the depolymerization of heparin. These drugs showed advantages in pharmacokinetics and convenience of administration.[1] There are various LMWHs with differences in biologic action, amount of pharmacologically active product, and results from clinical trials. Based on the manufacturing method, there are differences in the chemical structure of LMWHs. As an example, nadroparin is an LMWH produced by nitrous acid degradation, followed by sodium borohydride reduction, resulting in the characteristic 2,5-anhydro-D-mannose group at the reducing terminus (Figure 1).[2] As this variability introduces another level of structural complexity to the already diverse oligosaccharide sequence inherited from heparin, sophisticated analytical methods are needed to characterize LMWHs. From 2007 to 2008, acute side effects resulting in nearly 100 deaths could be associated with certain batches of heparin with semisynthetic oversulfated chondroitin sulfate contamination.[3] The heparin contamination crisis has shown that effective assays are required to ensure drug quality, by detecting known and unknown contaminants. Also, several biosimilar or generic versions of LMWHs were introduced to reduce treatment costs for patients. To ensure the equality of generic and innovator products, the equivalency of the sequence of oligosaccharide species needs to be proven.

In top-down approaches, time-consuming enzymatic digestion or degradation of oligosaccharides is circumvented, as the intact LMWH can be analyzed directly. In that way, different chromatographic approaches are usually combined with mass spectrometry (MS) to reveal the composition of LMWHs. Size exclusion chromatography (SEC) is a common technique for separation of different sizes of intact oligosaccharide chains. Nevertheless, SEC cannot be used to analyze the oligosaccharide composition or substitutions. Another method for characterization of LMWHs is ion-pairing revered-phase (IP-RP) chromatography coupled to MS, which has shown to be a specific and sensitive approach.[4] Recently, two-dimensional liquid chromatography (2D-LC), combined with quadrupole time-of-flight (Q-TOF) MS detection has proven to be a powerful tool in the analysis of oligosaccharide chains of LMWHs. Different degrees of polymerization (DPs) can be resolved by SEC in the first dimension (^{1}D), while IP-RP chromatography separates different oligosaccharide compositions in the second dimension (^{2}D).[5]

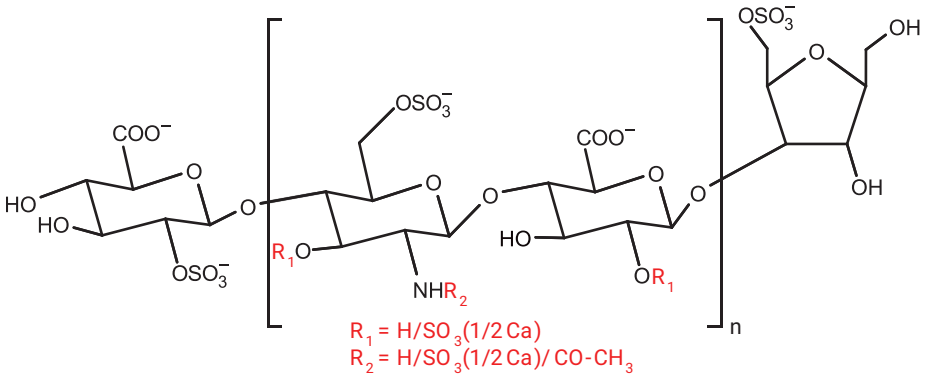

Figure 1. Chemical structure of nadroparin calcium.

The 1290 Infinity II Bio 2D-LC system combined with the 6546 LC/Q-TOF is an ideal tool to enable this analytical workflow. The flow path of 1290 Infinity II Bio LC modules consists of MP35N, a nickel-cobalt alloy, which can reduce potential corrosion from salt-containing buffers. SEC is usually performed with salt-containing buffer additives, that can also contaminate the electrospray ion source over time, and suppress the MS signal. To overcome this problem, the 2D-LC system can be used as an automated desalting tool using an extra diverter valve, that automatically diverts buffer from the ^{1}D to waste in the beginning of each ^{2}D run.[6] Agilent MassHunter Acquisition Software 11.0 enables easy control of both the 2D-LC system and acquisition of MS data in a single software component. The software processes 2D-LC/MS data such that it can easily be analyzed by Agilent MassHunter Qualitative Analysis software. This application note describes a workflow for 2D-LC/MS analysis of the LMWH nadroparin, using a combination of SEC and IP-RP chromatography combined with Q-TOF MS detection.

Experimental

Instrument

- 2x Agilent 1290 Infinity II Bio High-Speed Pump (G7132A) with Agilent Bio Jet Weaver mixer kit, 35 µL volume (G7132-68135)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Agilent InfinityLab Sample Thermostat (G4761A, option # 101)
- 2x Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with Agilent Quick-Connect Bio Heat-Exchanger, Standard Flow (G7116-60071, option # 065)

- 4x Agilent 1290 Infinity Valve Drive (G1170A) equipped with 1x Agilent InfinityLab Bio 2D-LC ASM Valve (G5643B), 2x Agilent Multiple Heart-Cutting Valves with biocompatible 40 µL loops and 1x biocompatible 2 position/10 port valve (G5641A)
- Agilent 1290 Infinity II DAD (G7117B) equipped with biocompatible InfinityLab Max-Light Cartridge Cell (G7117-60020)
- Agilent G6546A LC/Q-TOF with Agilent Dual Jet Stream ESI Source

Software

- Agilent MassHunter Acquisition Software 11.0
- Agilent MassHunter Qualitative Analysis Software 10.0
- Agilent 2D-LC Software for MassHunter (G2198AA)

Samples

Nadroparin calcium, European Pharmacopoeia Reference Standard (Sigma, Germany) was dissolved in water to 20 mg/mL.

Columns

- **First dimension (^{1}D):** 2x Agilent AdvanceBio SEC 200 Å, 4.6 × 300 mm, 1.9 µm (part number PL1580-5201)
- **Second dimension (^{2}D):** Agilent ZORBAX Extend 300 C18, 2.1 × 150 mm, 3.5 µm (part number 763750-902)

Method parameters

Parameter	Value
^{1}D Pump	
Flow	0.2 mL/min
Solvents	Isocratic, premixed solvents
Pressure Limit	600 bar
2D-LC Settings*	
Flow	0.4 mL/min
2D-LC Operation Mode	Time-based heart cut (HiRes mode, 4 × 3 s, loop filling: 25%)
Gradient Phases	Analysis: 34 min Equilibration: 4 min
Flush Gradient	Starting condition to 100 %B in 1 min, duration: 3 min
Diverter Valve	Switch time: 3 min
Pressure Limit	400 bar
Multisampler	
Injection Volume	4 µL
Needle Wash	6 s in flush port, wash solvent: water
Thermostat	4 °C
Column Compartments	
^{1}D and ^{2}D Temperature	30 °C

* Different sampling times, analytical gradients, and stop times were used for each DP.

Parameter	Value
Diode Array Detector (DAD)	
Wavelength	210 nm
Bandwidth	4 nm
Reference Wavelength	360 nm
Reference Bandwidth	100 nm
Peak Width	>0.2 min (4 s response time) (1.25 Hz)
Q-TOF	
Mass Range	*m/z* 500 to 2,500
Polarity	Negative
Data Storage	Profile
Acquisition Rate	2 spectra/s
Gas Temperature	200 °C
Gas Flow	9 L/min
Nebulizer	45 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	10 L/min
Capillary Voltage	5,000 V
Nozzle Voltage	2,000 V
Fragmentor	220 V
Skimmer	65 V
Quad Amu	*m/z* 400
Oct 1 RF Vpp	750 V

Table 1. Sampling times and gradients for each degree of polymerization (DP).

DP	Sampling Time (min)	Analytical Gradient	Stop Time (min)
6	30.07	0 min: 8 %B, 30 min: 28 %B, 32 min: 100 %B	189.34
8	29.33	0 min: 10 %B, 30 min: 30 %B, 32 min: 100 %B	188.60
10	28.61	0 min: 18 %B, 30 min: 38 %B, 32 min: 100 %B	187.88
12	27.96	0 min: 20 %B, 30 min: 40 %B, 32 min: 100 %B	187.23
14	27.33	0 min: 20 %B, 30 min: 40 %B, 32 min: 100 %B	186.60
16	26.76	0 min: 20 %B, 30 min: 40 %B, 32 min: 100 %B	186.03

Chemicals

- 1-Pentylamine (PTA) was obtained from Acros Organics, Belgium.
- Hexafluoroisopropanol (HFIP) and Ammonium Acetate (AA) were obtained from Merck, Germany.
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).
- For ^{2}D solvents, Agilent InfinityLab ultrapure LC/MS acetonitrile (ACN) (part number 5191-4496) and Agilent InfinityLab ultrapure LC/MS water (part number 5191-4498) were used.
- ^{1}D mobile phase: 50 mM AA in Water:ACN (9:1)
- ^{2}D mobile phase A: 0.15% HFIP and 0.2% PTA in Water
- ^{2}D mobile phase B: 0.15% HFIP and 0.2% PTA in Water/ACN (25/75)

Results and discussion

To achieve separation of different DPs occurring in nadroparin, two Agilent AdvanceBio SEC columns were connected in series in the first chromatographic dimension. Due to a total column length of 600 mm, the flow rate was kept at 200 µL/minute to achieve a moderate backpressure of approximately 420 bar. Relative standard deviations of peaks from different DPs were calculated based on 10 consecutive injections showing excellent reproducibility, with values between 0.039 and 0.088%. This allowed reliable time-based sampling of peaks using a high-resolution series. In Figure 2A, an overlay of 10 consecutive injections of nadroparin is depicted. High-resolution sampling was used to transfer several portions of the peak apex from different DPs to the ^{2}D column. Four consecutive cuts with a sampling time of 3 seconds were taken, resulting in a loop filling of 25% for each cut. Figure 2A highlights the different cut positions by cut markers for each DP of nadroparin. To retrace the correct cut position during data analysis, cut markers can easily be displayed and overlaid with the ^{1}D detector signal using the **extract chromatograms** functionality of the Agilent MassHunter Qualitative Analysis software. The quantity of transferred sample to the ^{2}D resulted in sufficient abundance in MS detection, eliminating signs of column overloading, such as strong peak tailing or splitting, which has been observed with higher percentages of loop-filling. As high-resolution sampling 2D-LC allows partial loop filling, use of the standard 2D-LC system setup, equipped with 40 µL loops, is possible for various 2D-LC applications. Therefore, the manual exchange of loops and extensive requalification of the 2D-LC system can often be avoided.

To resolve different compositions occurring in similar oligosaccharide chain lengths of nadroparin, IP-RP chromatography was used in the ^{2}D. Figure 2B depicts the total ion chromatogram for each IP-RP separation resulting from the first cut of each DP. Data analysis could be separately conducted for each 2D-LC cut, as the **file splitter** utility of the Agilent MassHunter Acquisition 11.0 software allowed splitting of 2D-LC/MS data according to the order of cuts from the ^{1}D. This enabled straightforward analysis

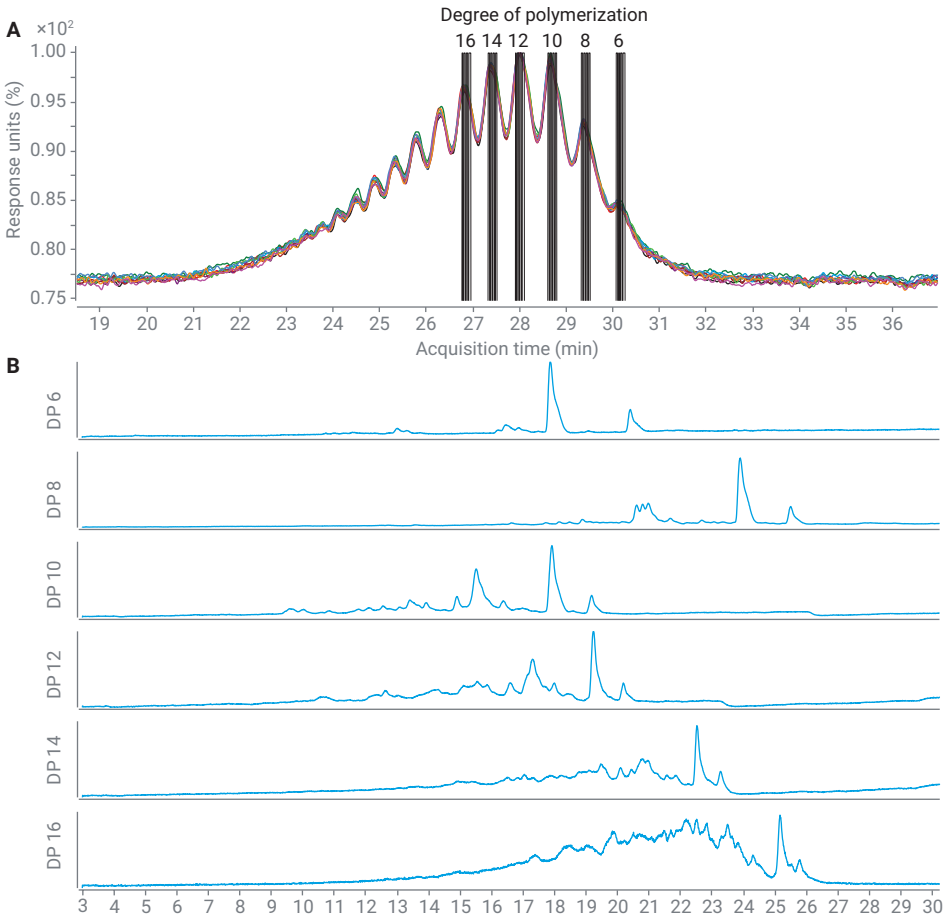

Figure 2. (A) Overlay of 10 consecutive runs resulting from SEC in the ^{1}D, showing the four cut markers for each DP. (B) Total ion chromatogram resulting from IP-RP analysis in the ^{2}D, showing the first cut of each high-resolution sampling series for each DP.

of complex MS data using MassHunter Qualitative Analysis software. Several different peaks could be resolved in the ^{2}D, indicating the occurrence of various oligosaccharide components of the same chain length in each DP. PTA and HFIP were used as IP reagents, as their potential as an optimized buffer system for IP-RP-MS analysis of heparin-derived oligosaccharides has been reported.[7] PTA resulted in superior separation compared to other linear aliphatic amines, while HFIP promotes protonation of PTA and has shown enhanced MS signal intensity. Also, adduct formation of PTA with sulfate groups protects oligosaccharides from loss of sulfate groups by in-source fragmentation, enabling the detection of the intact molecular ion.

In Figure 3A, the MS spectrum of the dodecasaccharide composition [6;5;1;0;17] resulting from high-resolution sampling of the DP12 peak is presented as an example. Standard nomenclature uses five numbers in squared brackets to describe oligosaccharides such as nadroparin, indicating the number of uronic acid residues, glucosamine residues, 2,5-anhydro-mannitol residues, acetyl, and sulfo groups. Several charge states of the oligosaccharide species were observed, while the threefold negatively charged state showed the highest abundance in this case. Many PTA adducts were observed for the oligosaccharide species, ranging from 7 to 12 PTA molecules per oligosaccharide chain (Figure 3B). Also, adduct formation with the mobile phase ingredient ACN was observed (marked with *). The asymmetric distribution of naturally occurring isotopes of the [M+10PTA-3H]$^{-3}$ ion of the dodecasaccharide composition [6;5;1;0;17] is shown in Figure 3C. The zoomed-in spectrum demonstrates that the Agilent G6546A LC/Q-TOF system showed excellent MS resolution, enabling the detection of the monoisotopic mass, as well as several isotopologues. A difference of approximately 1/3 Da was observed between the monoisotopic mass and further major isotopologues, confirming the threefold charge state of the molecule. For oligosaccharides with a higher DP and molecular mass, detection of the monoisotopic mass became more difficult, as the isotopic distribution was more symmetric and the signal intensity shifted toward the average mass. Nevertheless, several different oligosaccharide compositions were detected in accordance with literature[5] showing chain lengths from DP6 to DP16 (Table 2).

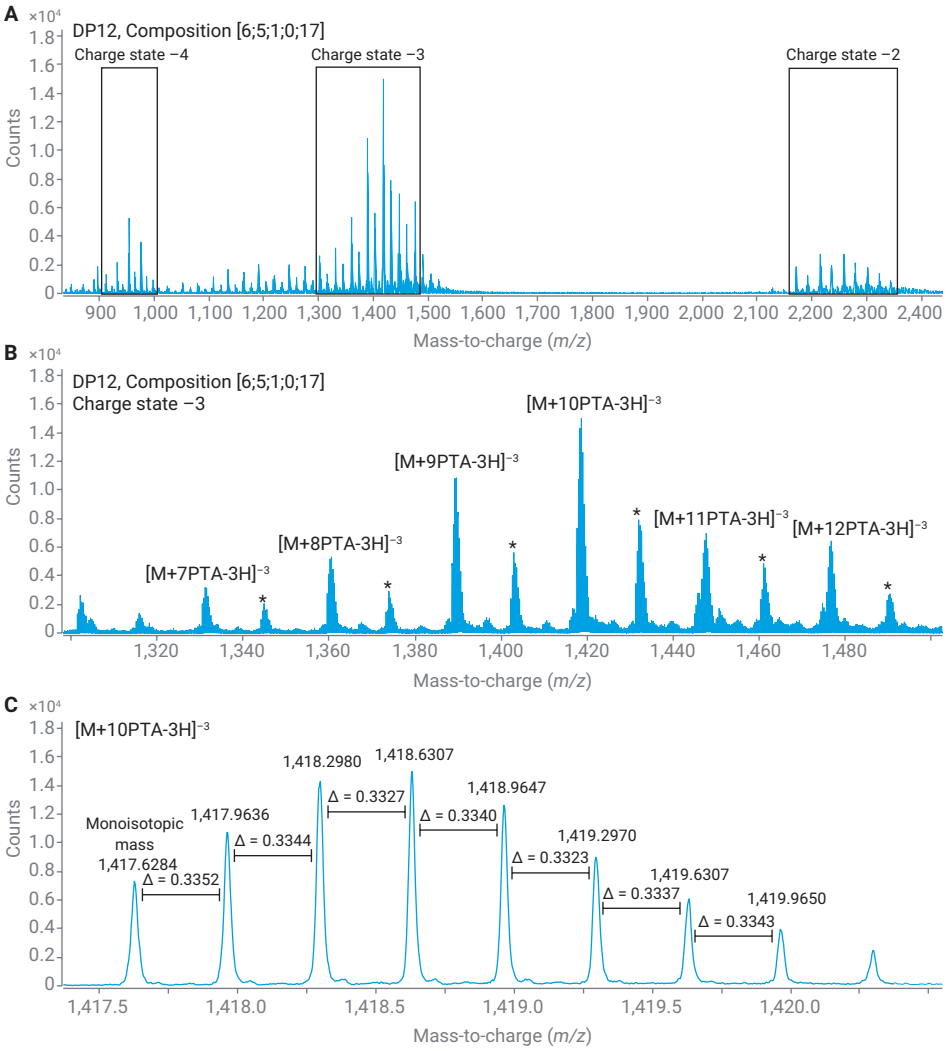

Figure 3. (A) MS spectrum of the dodecasaccharide composition [6;5;1;0;17] occurring in nadroparin, showing different charge states. (B) Highlighted MS spectrum showing different adducts with a threefold negative charge state (ACN adducts are marked with *). (C) Isotope distribution of the [M+10PTA-3H]$^{-3}$ ion species.

Table 2. Oligosaccharide compositions detected in nadroparin.

DP	Composition	Theoretical Mass (Da)
6	[4;3;1;1;6]	1,873.1500
6	[3;2;1;0;7]	1,574.0001
6	[3;3;0;3;6]	1,635.0858
6	[3;2;1;0;8]	1,653.9569
8	[4;3;1;0;11]	2,230.9282
8	[4;3;1;0;10]	2,150.9714
8	[4;3;1;3;10]	2,277.0031
8	[4;3;1;1;9]	2,113.0251
8	[4;3;1;0;9]	2,071.0146
8	[4;3;1;1;8]	2,033.0683
10	[5;4;1;1;11]	2,610.0397
10	[5;4;1;0;12]	2,647.9859
10	[5;4;1;1;12]	2,689.9965
10	[5;4;1;0;13]	2,727.9427
10	[5;4;1;0;14]	2,807.8996
12	[6;5;1;1;14]	3187.0110
12	[6;5;1;1;15]	3,266.9678
12	[6;5;1;0;16]	3,304.9141
12	[5;5;1;0;17]	3,208.8388
12	[6;5;1;0;17]	3,384.8709
14	[7;6;1;1;17]	3,763.9824
14	[7;6;1;1;18]	3,843.9392
14	[7;6;1;0;19]	3,881.8854
14	[7;6;1;0;20]	3,961.8422
16	[8;7;1;1;20]	4,340.9537
16	[8;7;1;1;21]	4,420.9105
16	[8;7;1;0;22]	4,458.8568
16	[8;7;1;0;23]	4,538.8136

High-resolution sampling 2D-LC/MS analysis of DP8 revealed six different oligosaccharide species, with the octasaccharide composition [4;3;1;0;11] showing by far the highest abundance (Figure 4). Several oligosaccharides with an identical MS spectrum and pattern of adduct ions eluted at different retention times in the ^{2}D. This observation can be attributed to the occurrence of differently ordered oligosaccharide chains with the same composition, and highlights the separation efficiency achieved by 2D-LC. For the ^{2}D separation of DP8, a gradient from 10 to 30 %B was used. For higher DPs, the gradient was shifted to higher percentages of solvent B to achieve sufficient resolution.

Figure 5 includes the mass spectra for all six different oligosaccharide compositions identified in DP8. The spectra are zoomed in to show adducts ions of the twofold negative charge state, as they showed the overall highest abundances in DP8. The oligosaccharide compositions and theoretical masses are presented in the top left corner of each spectrum, and were adapted from literature.[5] Based on the theoretical mass, different PTA and ACN adducts could be assigned to the individual pattern of MS spectrum peaks. As shown in Figure 5, the most abundant adduct ions contain fewer PTA molecules for oligosaccharide species with a lower number of sulfate groups, while species with a higher number of sulfate groups rather result in abundant adducts with a higher number of PTA molecules. This observation can be explained by the strong ion pair formation between positively charged PTA molecules from the mobile phase, and negatively charged sulfate groups of nadroparin. Due to the interaction of PTA with the stationary phase, an increased retention was observed for oligosaccharide species with higher numbers of sulfate groups. The last number in square brackets indicates the number of sulfate groups for each composition. Furthermore, the observed trends continued with higher DPs and are also in line with literature.[7]

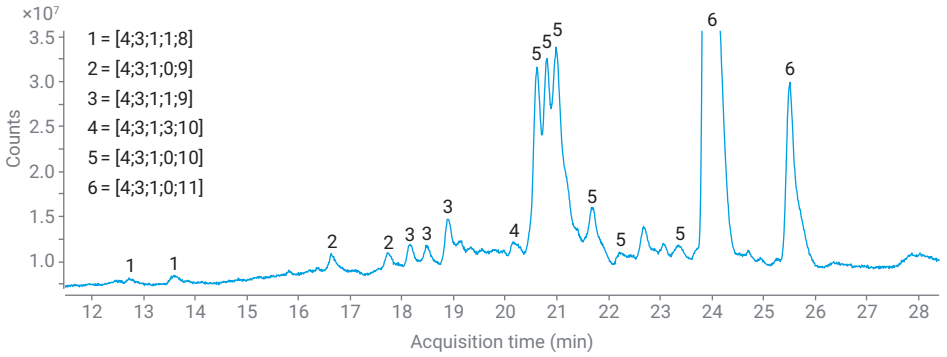

Figure 4. Total ion chromatogram of IP-RP analysis in the 2D showing the first cut from high-resolution sampling of DP8.

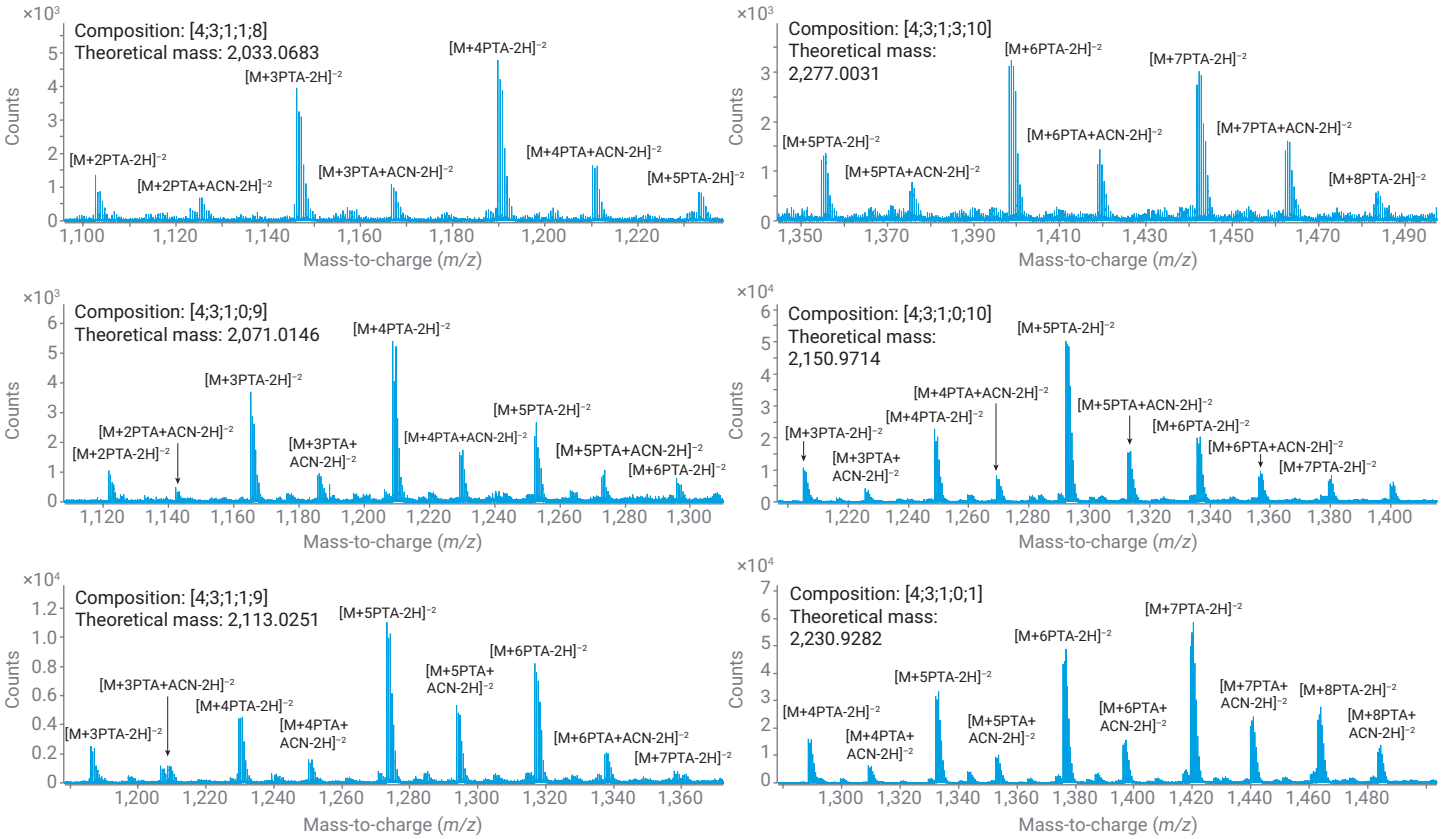

Figure 5. Mass spectra of oligosaccharide compositions occurring in DP8 of nadroparin.

Conclusion

Structural characterization of LMWHs is indispensable to ensure drug safety, by detection of known and unknown contaminations. Due to the heterogeneity and complexity of LMWHs, effective analytical tools are needed for monitoring of different oligosaccharide compositions. This application note describes a 2D-LC/MS workflow for characterization of the LMWH nadroparin. The Agilent 1290 Infinity II Bio 2D-LC System was used to couple two different separation techniques prior to MS analysis. The 2D-LC system was also used as an automated tool for desalting before MS analysis. For each DP resolved by SEC in the ^{1}D, several cuts were transferred to the ^{2}D by high-resolution sampling. SEC showed an excellent retention time precision, which is required for reliable time-based 2D-LC analysis. In the ^{2}D, several different oligosaccharide compositions with the same chain length could be separated by IP-RP chromatography. The Agilent 6546 LC/Q-TOF showed excellent MS resolution, and enabled the detection of reported oligosaccharide compositions occurring in nadroparin. The latest Agilent MassHunter Workstation Software enabled easy control of the 2D-LC/MS system in a single software platform, as well as straightforward analysis of the complex 2D-LC/MS data.

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