Comprehensive Characterization of Low Molecular Weight Heparins Using High Resolution Mass Spectrometry

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Low molecular weight heparin (LMWH) is class of antithrombotic drug known for their increased bioavailability and pharmacodynamics. The comprehensive characterization of LMWHs is important from the drug quality and safety aspects because of high risk of introduction of structurally related impurities due to the incomplete reactions or side reactions. Liquid Chromatography Mass spectrometric methods are the preferred methods for LMWH compositional analysis but are always challenged due to the presence of hundreds of peaks of broad molecular weights along with large number of structural isomers at each chain length having similar retention times. In addition, lack of suitable bioinformatics tools for fast and efficient spectral interpretation and detailed structure information on individual chains, makes it further challenging.

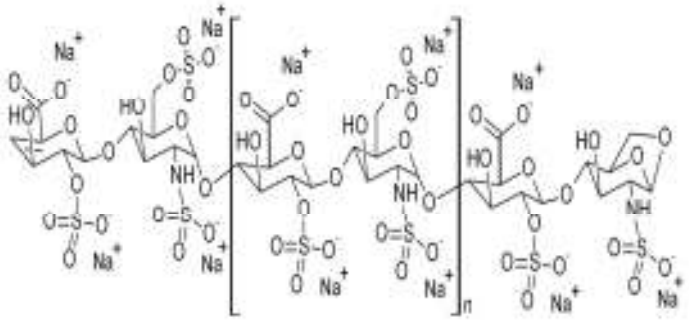

Figure 1. Structure of Enoxaparin Sodium

Figure 1: AB SCIEX TripleTOF® System

Key Benefits of TripleTOF® System for Analysis of LMW Heparins

1. Efficient data review and reporting with software provides high quality heparin molecular weight information from high resolution TOFMS data

2. Powerful LCMS reconstruction tools provide accurate mass assignment, even for highly heterogeneous heparin masses with repeated loss of SO4 and amine adducts.

3. The Structural Confirmation tool in PeakView for the identification and confirmation of the disaccharide units.

Experimental Design:

Sample Preparation

The LMWHs, enoxaparin sodium injection was purchased from local market. The Enoxaparin was diluted in mobile phase A as per the desired concentration.

Chromatography:

Rapid HPLC analysis of LMWHs was performed using a Shimadzu HPLC System on Agilent SB C18, 4.6 x 100 mm,1.8 um column. Two columns were connected in serial with a column coupler. The HPLC gradient is shown in Table 1. Solvent A consisted of 15mM hexylamine, and solvent B consisted of 15mM hexylamine with 75% Acetonitrile. The pH of both the mobile phases was adjusted to 7.0 with 0.1% Formic acid.

Time	%B
0.1	5
5	16
65	56
68	100
70	100
71	5
80	5

Data Processing:

Spectral deconvolution was performed using the Bio Tool Kit from PeakView® Software and Structural Elucidation tool was used for the confirmation of the Disaccharide unit.

Mass Spectrometry:

The MS analysis was performed on a TripleTOF® 5600 system (SCIEX) in negative electrospray ionization mode. The Information Data dependent Acquisition (IDA) workflow was performed as follows: MS scan acquired in high resolution mode using 250 ms accumulation time per spectrum followed by 10 MSMS scans of 100ms each. Each selected ion was then put on a dynamic exclusion list for 10 seconds. Collision energy (CE) was set to 40 with a collision energy spread (CES) of 15 V. Replicate runs were performed to assess run to run as well as day day to reproducibility.

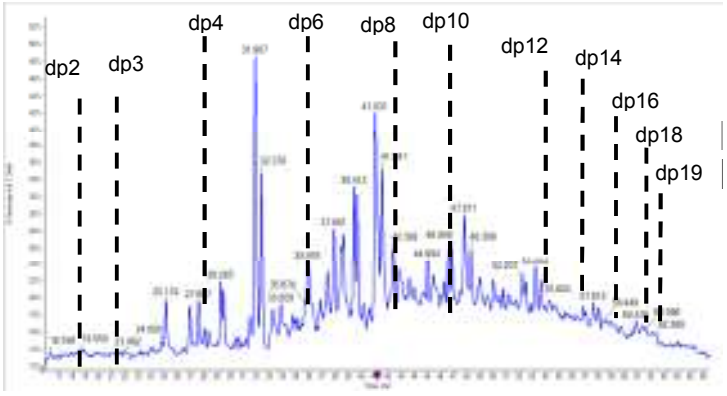

Figure 2. The fingerprinting profile of enoxaparin

Results and Discussion

The high quality TOFMS data with TripleTOF® in combination with chromatographic resolution resulted in the identification of enoxaparin oligosaccharides ranging from dp2 to dp20 with or without acetylation, variable sulfation degrees and high mass accuracy.

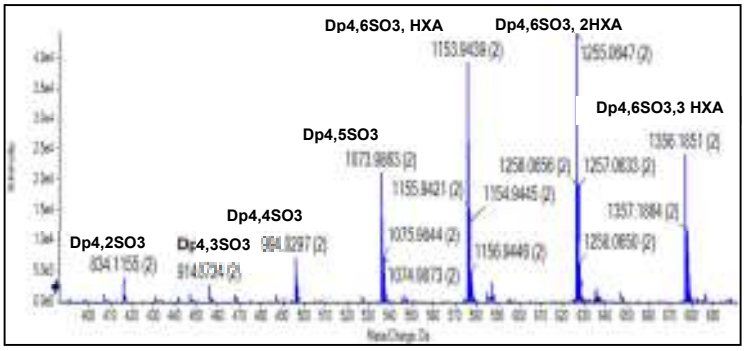

Figure 4. The TOFMS spectra of Enoxaparin at RT 31.9 min. showing variable degree of sulfation and hexyalmine adduct formation.

The mass spectra LMWHs are extremely complicated because of the heterogeneous LMWHs' structures, multiple charge states, as well as variable hexyalmine adduct formation. Data processing involved the automated LCMS reconstruction tool and then matching the experimental MWs to the theoretical MWs derived from the database containing all possible LMWH structures.

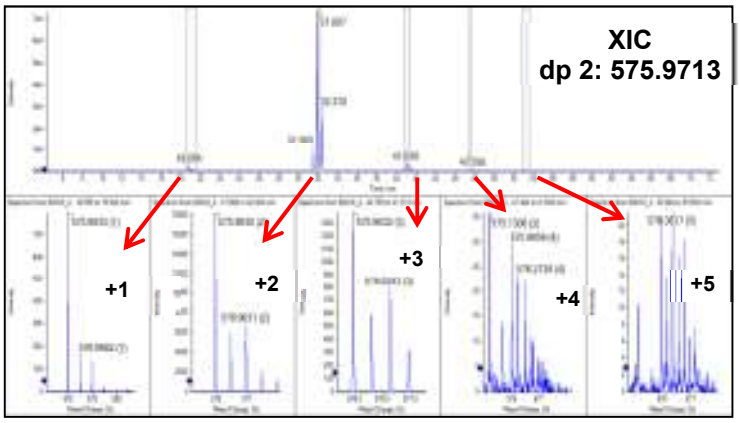

Figure 3.The Extracted Ion chromatogram of an Enoxaparin disaccharide (576.97) containing even numbers of units and unsaturated uronic acid at the non-reducing end. The presence of the same mass in multiple charge states with different retention times providing information on chain lengths and composition.

The extraction of the accurate mass of common disaccharides within the 50mDa window showed presence of the same mass in multiple charge states at different retention times providing information on chain lengths and composition. In addition to the oligosaccharides having the defined enoxaparin structures, some minor components such as odd number oligosaccharides and saturated enoxaparin, were also detected.

The number of potential isomers increases dramatically with the increase in the size of oligosaccharides, which in turn decreases the chromatographic resolution. The high scan speed with high sensitivity and resolution on a TripleTOF® platform helped in the detection of enoxaparin oligosaccharides with higher chain lengths, acetylation and higher degree of sulfation extending the detectable molecular weight range.

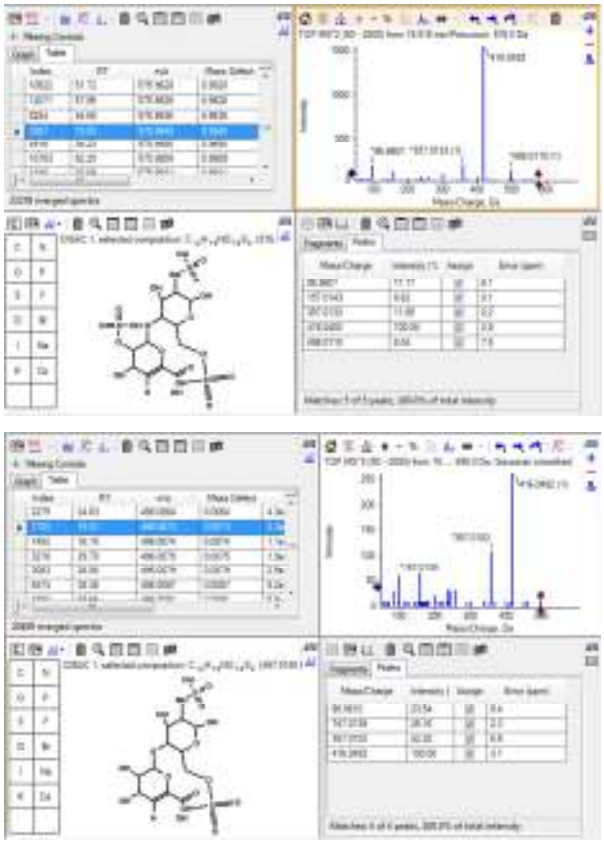

Figure 5: (A) Disac-1, 576.9- Fragmentation Pattern Comparison With the Structure obtained from the literature using structural elucidation tool in PeakView®. (B) Disac-2, m/z 497.052 - Loss of -SO3 group, verified from the theoretical fragmentation pattern.

Replicate analysis on different days showed highly reproducible results in terms of both retention times and areas which are important in comparing LMWH preparations and for evaluating lot-to-lot variability.

CONCLUSION:

1. The high scan speed with high sensitivity and resolution on TripleTOF® resulted in the identification of enoxaparin oligosaccharides with higher chain lengths

2. The XIC manager and LCMS reconstruction tools in PeakView® provided accurate mass assignment, even for highly heterogeneous heparin masses.

3. Highly reproducible results were obtained in terms of both retention times and areas which are important for evaluating various LMWH preparations and lot-to-lot variability.

4. The present study highlights the advantages of High performance fast scanning TripleTOF® system with high resolution chromatography for in depth characterization of the oligosaccharide composition of LMWH.

REFERENCES:

1. Daoyuan Li, Lequan Chic, Lan Jin , Xiaohui Xu, Xuzhao Du, Shengli Ji , Lianli Chia, Trethewey, R. N. Carbohydrate Polymers 99 (2014) 339– 344.

2. Adam M. Brustkern, Lucinda F. Buhse, Moheb Nasr, Ali Al-Hakim, and David A. Keire. Anal. Chem. 2010, 82, 9865–9870.

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