Structural Analysis of Heparin Derived Oligosaccharides by Ion-Pair Reversed-phase Liquid Chromatography Coupled with Electrospray Ionization Time-of-Flight Mass Spectrometry

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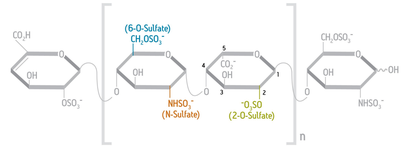
OVERVIEW

- The work covers the development of two analytical methods for the analysis of heparin and heparin derived oligosaccharides.
- An ion-pair reversed-phase ultra-performance liquid chromatography method was developed for rapid analysis of heparin oligosaccharides coupled with electrospray ionization time-of-flight mass spectrometry [(IPRP)-UPLC ESI/TOF-MS].
- An anion exchange chromatography (AXC) method was developed to separate and quantify intact heparin and oversulfated chondroitin sulfate (OSCS).
- The IPRP-UPLC ESI/TOF-MS method combines a high-resolution LC separation with the accurate mass measurement of Q-Tof mass spectrometry to yield in-depth physicochemical characterization of heparin oligosaccharides.
- The anion exchange chromatography method achieves complete resolution between heparin and OSCS, and less than 1% of OSCS in overall content is successfully quantified by UV absorbance.

The UPLC-Mass Spectrometry System Used in the Method Development for Heparin Oligosaccharide Analysis

Structures of Heparin Oligosaccharides Derived from Heparinase Digestion of Bovine Heparin



EXPERIMENTAL METHODS

♦ Samples:
- dp6/dp8/dp10 oligosaccharides: V-Labs, Inc., LA, USA
- Heparin std & heparin system test std:US Pharmacopeia

♦ IPRP-UPLC Conditions
- LC System: Waters ACQUITY UPLC® System
- Column: ACQUITY UPLC™ BEH C18 2.1x150mm, 1.7µm
- Column Temperature: 45 °C
- Mobile Phases
  - A: 15 mM Hexylamine (HXA) /100mM 1,1,1,3,3,3,-hexafluoro 2-propanol (HFIP) aqueous solution
  - B: 15 mM HXA/100mM HFIP in 75/25 (v/v) ACN/H_2O
- LC Gradient: 25%B – 60% B in 10 minutes

♦ MS Conditions

Mass Spectrometer:	Waters Q-Tof Premier™	
Acquisition Mode:	Negative Ion Mode	
Capillary Voltage	3.0 kV	CE: 2V
Source Temperature:	120 °C	Cone Voltage: 15V
Extractor Voltage:	1 V	Scan Time: 0.6 s
m/z Range:	350 – 1900	

♦ Anion Exchange Chromatography Conditions
- LC System: Waters Alliance® Bioseparation (AllianceBIO)
- Column: Waters Spherisorb® 5µm SAX, 4.0X250mm
- Column Temperature: 40 °C
- Mobile Phases
  - A: 50 mM NaH_2PO_4 (pH 2.5)
  - B: 50 mM NaH_2PO_4 + 2.0 M $NaClO_4$ (pH 2.5)
- LC Gradient: 10%B – 90% B in 10 minutes

♦ UV Detections
- Waters 2998 Photodiode Array (PDA) Detector
- Wavelength: 190—400 nm

Chromatographic Separations of Heparin Oligosaccharide Isomers/Mixture

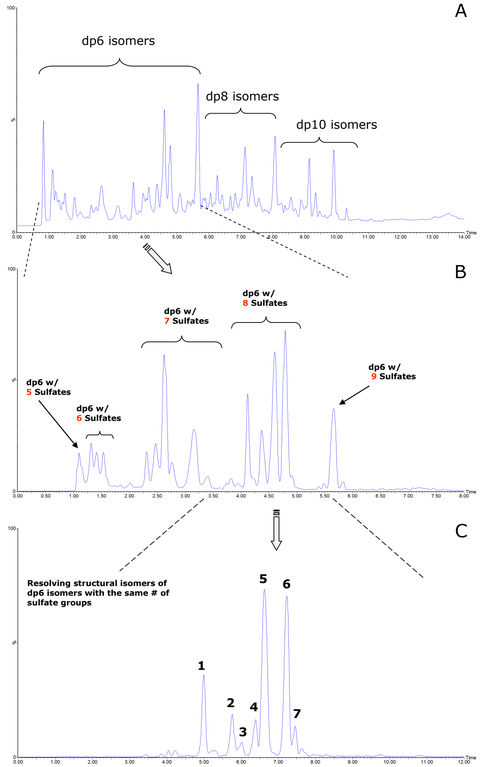


Figure 1. Chromatographic separations of a mixture of dp6/dp8/dp10 heparin derived oligosaccharides:
(A) TIC chromatogram showing the separation of different oligosaccharide classes;
(B) Mass chromatogram of dp6 oligosaccharides containing 5, 6, 7, 8 and 9 sulfate groups;
(C) Extracted ion chromatogram (*m/z=925.59*) showing the chromatographic resolution of octa-sulfated dp6 isomers. There are totally nine (9) possible isomeric structures for this compound.

Accurate Mass Measurement to Determine the Molecular Entities of Heparin Isomers

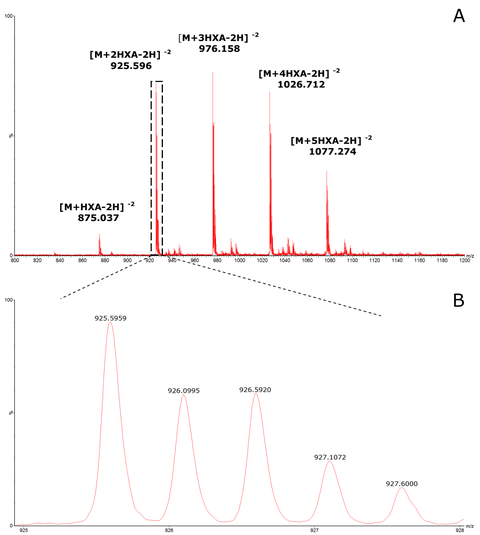


Figure 2. (A) Electrospray mass spectrum of a heparin-derived oligosaccharide: octa-sulfated dp6 isomer, demonstrating no sulfate group losses during MS analysis; (B) Fully resolved isotopic distribution of the MS peak at *m/z* 925.596, enabling accurate mass measurement on the mono-isotopic peak for isomer identification.

Oligosaccharide	Elemental Composition	Monoisotopic ions observed in ESI-MS				
dp6		[M + HXA - 2H]$^{2-}$	[M + 2HXA - 2H]$^{2-}$	[M + 3HXA - 2H]$^{2-}$	[M + 4HXA - 2H]$^{2-}$	[M + 5HXA - 2H]$^{2-}$
nona-sulfated	C36 H57 N3 O57 S9	915.0100	965.5703	1016.1306	1066.6910	1117.2513
octa-sulfated	C36 H58 N3 O54 S8	875.0316	925.5919	976.1522	1026.7125	1077.2729
hepta-sulfated	C36 H59 N3 O51 S7	835.0532	885.6135	936.1738	986.7341	1037.2944
hexa-sulfated	C36 H60 N3 O54 S6	795.0748	845.6351	896.1954	946.7557	997.3160
dp8		[M + 4HXA - 2H]$^{2-}$	[M + 5HXA - 2H]$^{2-}$	[M + 6HXA - 2H]$^{2-}$	[M + 7HXA - 2H]$^{2-}$	[M + 8HXA - 2H]$^{2-}$
dodeca-sulfated	C48 H76 N4 O76 S12	1405.7370	1456.2973	1506.8576	1557.4179	1607.9783
undeca-sulfated	C48 H76 N4 O73 S11	1315.20	1365.76	1416.32	1466.88	1517.44
deca-sulfated	C48 H76 N4 O70 S10	1224.6596	1275.2199	1325.7802	1376.3405	1426.9008
dp10		[M + 5HXA - 3H]$^{3-}$	[M + 6HXA - 3H]$^{3-}$	[M + 7HXA - 3H]$^{3-}$	[M + 8HXA - 3H]$^{3-}$	[M + 9HXA - 3H]$^{3-}$
pentadeca-sulfated	C60 H95 N5 O95 S15	1129.1459	1162.8528	1196.5597	1230.2665	1263.9734
tetradeca-sulfated	C60 H95 N5 O92 S14	1102.49	1136.20	1169.91	1203.61	1237.32

Table I. All of heparin derived oligosaccharides identified from the dp6/dp8/dp10 mixture in this study.

Developing an Anion Exchange Chromatography Method for Analysis of Intact Heparin Containing Oversulfated Chondroitin Sulfate (OSCS)

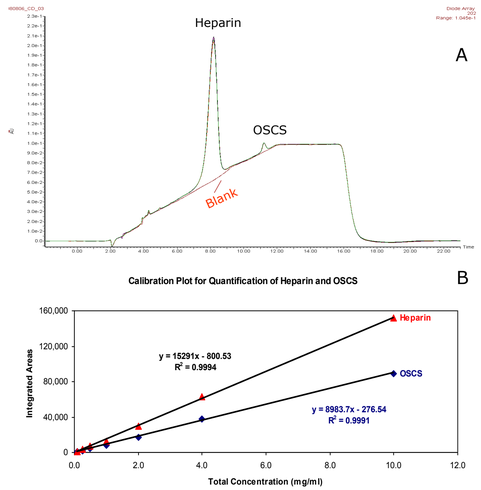


Figure 3. (A) LC-UV chromatograms show the separation of the heparin from OSCS (~1% of heparin concentration). Replicate injections (3) are shown to demonstrate the reproducibility of the separation; (B) Calibration plots for quantifications of heparin and oversulfated chondroitin sulfate (OSCS). At least 2-order of magnitude of linear dynamic range is obtained for each analyte.

CONCLUSIONS

- A simple, rapid and sensitive IPRP-UPLC/TOF MS method was developed for analysis of heparin derived isomers. Structural information such as molecular weight, number of sulfate groups, and composition of disaccharide blocks of heparin can be reliably obtained using the method.
- An anion exchange chromatography method was developed for separation and quantification of intact heparin and oversulfated chondroitin sulfate. The results demonstrate the method not only generates highly reproducible and fast separations (10 minutes) but also quantifies OSCS with abundance less than 1% of overall contents.

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