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UNIVERSITY OF CALIFORNIA
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Advancing Analytical Methods for Characterization of Anionic Carbohydrate
Biopolymers

A Dissertation submitted in partial satisfaction
of the requirement for the degree of

Doctor of Philosophy

in

Chemistry

by

Derek Joseph Langeslay

June 2013

Dissertation Committee:

Dr. Cynthia K. Larive, Chairperson
Dr. Wenwan Zhong
Dr. Yinsheng Wang

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The Dissertation of Derek Joseph Langeslay is approved:

Committee Chairperson

University of California, Riverside

Acknowledgements

It has been a long road leading me to where I am today. Somewhere around the 5th grade I remember writing a short paper on what I wanted to be when I grew up. Even before I knew what it meant exactly, I knew I wanted to be a scientist. Today I am closer to that goal than ever. Though this path has been arduous, I have had many people help steer me along the way. As a child my parents encouraged my curiosity of the natural world by buying me microscope kits and encouraging my curiosity of science and nature. For that I would like to first and foremost thank my parents Richard Langeslay and Valerie Weiss. Another big influence in my life has been my brother Ryan Langeslay. As a fellow chemist, he has provided me with inspiration, direction and connections which have led to several chemist positions throughout the years.

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Acknowledgement of Copyright

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ABSTRACT OF THE DISSERTATION

Advancing Analytical Methods for Characterization of Anionic Carbohydrate Biopolymers

by

Derek Joseph Langeslay

Doctor of Philosophy, Graduate Program in Chemistry
University of California, Riverside, June 2013
Dr. Cynthia K. Larive, Chairperson

The focus of this dissertation is on the development of improved analytical methods for the characterization of anionic carbohydrate biopolymers. Our goal is to extract important information from complex mixtures of heterogeneous polysaccharides by characterizing their substituent oligosaccharides in terms of monosaccharide composition and primary and secondary structure. This work focuses on the application of two major analytical platforms: spectroscopy and chromatography.

The development of new nuclear magnetic resonance spectroscopy (NMR) tools for the characterization of the biologically active carbohydrates heparin and heparan sulfate (HS) is presented in Chapters 2-4. These biopolymers are members of a special class of nitrogen containing polysaccharides called glycosaminoglycans (GAGs). Our discovery of experimental parameters for detection of the ^1H and ^{15}N resonances of the sulfamate (NHSO_3^-) groups of the *N*-sulfoglucosamine residues of heparin and HS was an important breakthrough and demonstrated the sensitivity of these chemical shifts to local structure. Evaluation of the exchange kinetics of the sulfamate group protons with the bulk aqueous solvent allowed us to explore the relationship between primary and

secondary structure in heparin oligosaccharides and led to our identification of the first solution state hydrogen bond between a sulfamate group NH proton and the adjacent 3-*O*-sulfate moiety of the heparin drug Arixtra (Fonduparinux sodium). Generation of a ^1H and ^{15}N chemical shift library for a series of heparin-derived oligosaccharides and chemically modified heparins allowed the assignment of the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC correlations in spectra measured for unfractionated and low-molecular-weight heparins (LMWH) as well as commercial and human-derived ^{15}N -enriched HS isolated from cell culture.

The analysis of LMWH samples by reversed-phase ion-pairing ultraperformance liquid chromatography with detection by mass spectrometry (RPIP-UPLC-MS) is described in Chapter 5. This method utilizes volatile amphiphilic ion-pairing reagents to retain anionic oligosaccharides on a hydrophobic stationary phase while facilitating detection with the information rich method of mass spectrometry. With this platform we were able to separate individual components of complex LMWH mixtures providing a fingerprint of different drug preparations. The potential of this approach for quality assurance applications was illustrated by the comparison of the relative abundances of individual oligosaccharides highlighting slight differences between LMWH samples.

Table of Contents

Acknowledgements	iv
Copyright Acknowledgements	vi
Abstract of the Dissertation	vii
Table of Contents	ix
List of Figures	xiv
List of Tables	xxi
CHAPTER ONE: Introduction	1
1.1 Glycosaminoglycans	2
1.2 Heparin and heparan sulfate	5
1.3 Biological significance	5
1.4 Depolymerization strategies	9
1.4.1 Enzymatic depolymerization.	9
1.4.2 Chemical depolymerization.	10
1.5 Separation of oligosaccharides	12
1.5.1 Size-exclusion chromatography.	14
1.5.2 Ion- Exchange Chromatography.	15
1.5.3 Reversed-phase ion-pair - ultraperformance liquid chromatography (RPIP-UPLC).	19
1.6 Mass Spectrometry	20
1.6.1 Negative-mode electrospray ionization mass spectrometry.	20

1.6.2 Matrix-assisted laser desorption ionization mass spectrometry.	21
1.6.3 Tandem Mass Spectrometry.	22
1.7 Nuclear magnetic resonance spectroscopy	23
1.7.1 ^1H NMR spectroscopy analysis of glycosaminoglycans.	24
1.7.2 Homonuclear two-dimensional NMR spectroscopy.	26
1.7.3 Heteronuclear two-dimensional NMR spectroscopy.	33
1.8. Summary	39
1.9. References	40
CHAPTER TWO: Detection and assignment of sulfamate	49
^1H and ^{15}N NMR resonances of <i>N</i>-sulfo-glucosamine	
2.1 Introduction	50
2.2 Methods and Materials	53
2.2.1 Materials and reagents.	53
2.2.2. NMR parameters.	53
2.3. Results and Discussion	59
2.3.1. Detection of sulfamate resonances by ^1H NMR.	59
2.3.2. Detection of sulfamate resonances by [^1H , ^{15}N] HSQC NMR.	65
2.3.3. ^1H and ^{15}N NMR measurements of the sulfamate groups of the pentasaccharide Arixtra.	67

2.3.4. [^1H , ^{15}N] HSQC-TOCSY as a potential tool for heparin characterization.	70
2.3.5. Comparison of results obtained with the [^1H , ^{15}N] IMPACT- HNMBBC and [^1H , ^{15}N] HSQC-TOCSY experiments.	77
2.4. Conclusions	88
2.5. References	89
CHAPTER THREE: Sulfamate proton solvent exchange in heparin oligosaccharides – Evidence for a persistent hydrogen bond in the antithrombin-binding pentasaccharide Arixtra	93
3.1. Introduction	94
3.2. Methods and Materials	96
3.2.1. Materials and reagents.	96
3.2.2. NMR parameters.	98
3.2.3. Molecular dynamics simulation.	101
3.3. Results and Discussion	103
3.3.1. pH dependence of the sulfamate ^1H resonance line width.	104
3.3.2. Sulfamate NH temperature coefficients.	109
3.3.3. Calculation of the energy barrier for sulfamate proton solvent exchange.	116
3.4.4. Molecular dynamics simulation.	118
3.4. Conclusions	125
3.5. References	126

CHAPTER FOUR: Characterizing the microstructure of heparin and heparan sulfate using <i>N</i>-sulfo-glucosamine ^1H and ^{15}N NMR chemical shift analysis	132
4.1. Introduction	133
4.2. Methods and Materials	135
4.2.1. Materials and reagents.	135
4.2.2. Heparinase-I digestion.	136
4.2.3. Heparinase-III digestion.	136
4.2.4. Isolation of oligosaccharide standards.	136
4.2.5. Isolation of HS from cell culture.	138
4.2.6. NMR parameters.	139
4.3. Results and Discussion	141
4.3.1. Defining GlcNS microstructures in heparin and HS.	141
4.3.2. Chemical shift analysis of GlcNS containing oligosaccharide standards.	142
4.3.3 Chemical shifts of the terminal GlcNS residues.	143
4.3.4. Effect of uronic acid 2-O-sulfation on GlcNS chemical shifts.	148
4.3.5. Effect of 3-O-sulfation on GlcNS chemical shifts.	150
4.3.6. HSQC-TOCSY correlations confirm resonance assignments.	151
4.3.7. Effect of 6-O-sulfation on GlcNS chemical shifts.	155
4.4. Conclusions	158

4.5. References	161
CHAPTER FIVE: Reversed-phase ion-pair UPLC-MS for fingerprinting low-molecular-weight heparins	164
5.1. Introduction	165
5.2. Materials and Methods	173
5.2.1. Materials and reagents.	173
5.2.2. Size-exclusion chromatography profiling of LMWH.	173
5.2.3. UPLC separations.	173
5.2.4. Mass spectrometry.	174
5.3. Results and Discussion	176
5.3.1. Mobile phase selection.	176
5.3.2. Comparison of DBA, TrBA, and PTA separations.	178
5.3.4. Analysis time.	183
5.3.5. Reproducibility.	185
5.3.6. Mass spectral assignments and interpretation.	186
5.3.7. Comparison of tinzaparin and enoxaparin RPIP-UPLC-MS fingerprints.	192
5.3.8. Comparison of the RPIP-UPLC-MS fingerprints of Clexane and the U.S.P. and E.D.Q.M. enoxaparin reference standards.	194
5.4. Conclusions	199
5.5. References	201
CHAPTER SIX: Conclusions and Future Directions	206

6.1 Conclusions	206
6.2 Future Directions	208
6.3 References	213
Appendix 1	215
Appendix 2	221

LIST OF FIGURES

Figure 1.1. Haworth projections of GAG structures. A) HA, B) CS, C) DS, D) HS and heparin, where R= H or SO ₃ ⁻ and R'= H, SO ₃ ⁻ or COCH ₃ .	4
Figure 1.2. An illustration of the biosynthetic process converting the precursor <i>N</i> -acetylheparosan into heparin.	6
Figure 1.3. Antithrombotic heparin mimetic drug Arixtra with Roman numeral designations for each monosaccharide.	8
Figure 1.4. Illustration of heparin lyase I, II, and III depolymerization products and substrate specificity.	11
Figure 1.5. Illustration of chemical depolymerization products and reaction specificities.	13
Figure 1.6. SEC chromatogram of 1g of heparinase-I digested porcine mucosal heparin separated on a 3 x 200 cm packed column. Numbers represent the number of monosaccharide residues comprising the depolymerization product. Product concentration was determined using the UV absorbance at 232 nm and a molar extinction coefficient of 5400 M ⁻¹ cm ⁻¹ .	16

Figure 1.7.	18
SAX chromatogram of hexasaccharide fraction (peak 6) of heparinase-I digested porcine mucosal heparin isolated from the SEC separation illustrated in Figure 1.6. The asterisk denotes hexasaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-IdoA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-IdoA}(2\text{S})\text{-GlcNS}(6\text{S})$, which is discussed in Chapter 4.	
Figure 1.8.	27
^1H NMR spectra in order of increasing structural complexity (A-F), (A) hyaluronic acid (B) chondroitin sulfate, (C) dermatan sulfate, (D) heparan sulfate, (E) unfractionated heparin, (F) enoxaparin. (G) the pure oligosaccharide Arixtra. The structure of the Arixtra pentasaccharide is shown in Figure 1.3.	
Figure 1.9.	29
TOCSY spectrum measured for Arixtra in D_2O using presaturation with residue and proton assignments labeled.	
Figure 1.10.	31
Expansion of the DQF-COSY spectrum of Arixtra showing the cross peak connecting H1 and H2 of the GlcNS6S(V) residue, A) processed in phase-sensitive mode in F2 and F1, B) processed in phase sensitive mode in F2 and magnitude mode in F1.	
Figure 1.11.	34
ROESY spectrum measured for Arixtra in D_2O using presaturation with through space correlations labeled.	
Figure 1.12.	38
The anomeric region of the $[^1\text{H}, ^{13}\text{C}]$ HSQC spectrum of enoxaparin with assignments labeled, where Man = mannose, and 1,6 an =1,6 anhydro. Peaks labeled ΔUA reflect the unsaturated uronic acid resulting from depolymerization and those labeled Gal/Xyl are due to the galactose and xylose residues of the linking region.	
Figure 2.1.	60
Selected regions of the ^1H NMR spectra of <i>N</i> -sulfo- <i>D</i> -glucosamine (GlcNS) measured as a function of pH in 90% $\text{H}_2\text{O}/10\%$ D_2O at 25 °C using WATERGATE W5 solvent suppression. The sulfamate proton resonances of the GlcNS α (5.34 ppm) and β (5.79 ppm) anomers are indicated. The asterisk marks the carbon-bound proton resonance of an impurity.	

Figure 2.2.	62
Selected regions of the ^1H NMR spectra of <i>N</i> -sulfo- <i>D</i> -glucosamine (GlcNS) measured as a function of pH in 90% H_2O /10% D_2O at 25 °C using WATERGATEW5 solvent suppression. A) The spectral region containing the GlcNS H2 resonances showing the effect of the sulfamate proton chemical exchange. B) A 5-fold scale enhancement of the H2 resonance of the β anomer.	
Figure 2.3.	64
Proton exchange mechanisms. A) acid-catalyzed proton exchange, B) base-catalyzed proton exchange, C) imidic acid proton exchange mechanism. In (A) and (B) X can be C or S. In (C) X = C.	
Figure 2.4.	66
$[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC spectrum of GlcNS at pH 7.5 and 5 °C. The asterisk marks the carbon-bound proton resonance of an impurity.	
Figure 2.5.	69
A) Structure of Arixtra. B) $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC spectrum of Arixtra in 90% H_2O /10% D_2O in 20 mM phosphate buffer at pH 8.2 and 5 °C. The ^1H WATERGATE W5 spectrum is plotted at the top of the contour map showing the carbon-bound anomeric proton resonances of GlcNS residues I and III in addition to the sulfamate proton resonances. C) $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC spectrum of Arixtra measured without ^{15}N decoupling. The measured $^1J_{(\text{HN})}$ values are displayed for each sulfamate group.	
Figure 2.6.	71
$[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC-TOCSY spectrum of 100 mM GlcNS and GlcNAc in 90% H_2O /10% D_2O at 5 °C and pH 7.48.	
Figure 2.7.	74
$[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC-TOCSY spectrum measured for a 40 mM Arixtra solution in 90% H_2O /10% D_2O containing 25 mM phosphate at pH 8.10 and 5 °C.	
Figure 2.8.	78
Pulse sequence for the acquisition of $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ IMPACT-HMBC spectra presented in this research.	
Figure 2.9.	80
$[\text{}^1\text{H}, \text{}^{15}\text{N}]$ IMPACT-HNMBC spectrum of 100 mM GlcNS and 100 mM GlcNAc in D_2O solution at 25 °C.	

Figure 2.10. [¹ H, ¹⁵ N] IMPACT-HNMBC spectrum of Arixtra in D ₂ O analyzed at 25 °C in 300 μL using a 5 mm D ₂ O-matched Shigemi NMR tube.	82
Figure 2.11. [¹ H, ¹⁵ N] IMPACT-HNMBC of Arixtra measured at 800 MHz using a cold probe.	83
Figure 2.12. [¹ H, ¹⁵ N] IMPACT-HMBC spectrum of 150 mM GlcN(3S) in 100% D ₂ O with 10 mM phosphate at pD 7.0 and 25 °C.	86
Figure 3.1. Structures of compounds used in this study. A) <i>N</i> -sulfoglucosamine showing the structures of both the α and β anomeric forms, B) heparin disaccharide ΔUA(2S)-GlcNS(6S) where ΔUA indicates the unsaturated uronic acid residue introduced by heparin lyase cleavage, C) heparin tetrasaccharide ΔUA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S), D) the Arixtra pentasaccharide. For the structures in (B) and (C) the presence of the α and β anomers is indicated by a wavy bond for the reducing end anomeric OH group.	97
Figure 3.2. Example simulation of an Arixtra spectrum measured at 283 K. A) Simulated spectrum, B) measured spectrum, C) residual (experimental – simulated) spectrum plotted at a 20-fold expansion of the vertical scale.	102
Figure 3.3. Variation of sulfamate ¹ H resonance line width as a function of pH for A) the α and β anomers of GlcNS, B) the disaccharide ΔUA(2S)-GlcNS(6S) α anomer, C) Arixtra measured at 25 °C, D) Arixtra GlcNS(III) measured at 37 °C.	106
Figure 3.4. Selected ¹ H NMR spectra measured for Arixtra as a function of temperature. The three NH resonances are labeled according to the positions of the GlcNS residues in the Arixtra structure (Figure 3.1). The doublets at 5.499 and 5.625 ppm are due to the carbon bound anomeric protons (H1) of GlcNS(III) and GlcNS(I), respectively.	111

Figure 3.5. 112

Graphs used to calculate ^1H temperature coefficients, $\Delta\delta/\Delta T$, for the sulfamate groups of A) GlcNS, B) heparin disaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})$, C) heparin tetrasaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-IdoA}(2\text{S})\text{-GlcNS}(6\text{S})$, D) Arixtra. Structures are given in Figure 3.1.

Figure 3.6. 113

TOCSY-W5 spectrum used for the assignment of the sulfamate group ^1H NMR resonances of the heparin tetrasaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-IdoA}(2\text{S})\text{-GlcNS}(6\text{S})$ where F2 is plotted along the horizontal axis. The numbers refer to the sulfamate ^1H resonance of the internal (II) and reducing end (IV- α) GlcNS(6S) residues, as in Figure 3.1. The intense cross peaks at 4.816 ppm in F1 arise from chemical exchange of the sulfamate NH protons with water during the evolution period.

Figure 3.7. 119

Plots used to determine the activation energy barrier, $\Delta G^\ddagger$, for the chemical exchange of the sulfamate protons with water for A) GlcNS(α), B) GlcNS(β), C) heparin disaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-(}\alpha\text{)}$, D) Arixtra GlcNS(I) at pH 8.2, E) Arixtra GlcNS(III) at pH 8.2, F) Arixtra GlcNS(V) at pH 8.2, G) Arixtra GlcNS(I) at pH 7.4, H) Arixtra GlcNS(III) at pH 7.4. The Arixtra GlcNS(V) sulfamate proton exchanged too rapidly at pH 7.4 to acquire enough data points for a reliable exponential fit. Points correspond to the NH resonance line widths at half height determined by nonlinear least squares fitting of the experimental spectra with a Lorentzian line shape. The red line corresponds to the exponential fit of the data to the Eyring-Polanyi equation.

Figure 3.8. 123

Representative structures from the 5 ns molecular dynamics simulation of the Arixtra pentasaccharide showing IdoA(2S) in the $^2\text{S}_0$ (A) and $^1\text{C}_4$ (B) conformations. The distances noted represent the average distance between the sulfamate protons and the oxygen of the sulfate at the 3-O position.

Figure 3.9. 127

Representative structures from the 5 ns molecular dynamics simulation of the Arixtra pentasaccharide showing IdoA(2S) in the $^2\text{S}_0$ (A) and $^1\text{C}_4$ (B) conformations. The hydrogen bonds were calculated for each conformer using a heavy atom distance cutoff between the donor and the acceptor set of 3.5 Å and an angle cutoff of 120°. Percentage of structures in which the hydrogen bond is present is noted.

Figure 4.1. [¹ H, ¹⁵ N] HSQC spectra of GAG samples: a) enoxaparin, b) porcine intestinal mucosa heparin, c) bovine lung heparin, d) Celsus porcine HS, and e) ¹⁵ N-enriched HEK293T-derived HS. Spectra were measured at 20° C in 90% H ₂ O/10% D ₂ O.	144
Figure 4.2. Structures of oligosaccharides used for chemical shift analysis. The abbreviations for each compound are also presented in Table 4.2: A) Di. B) Tetra-1. C) Tetra-2. D) Tetra-3. E) Tetra-4. F) Tetra-5. G) Tetra-6. H) Tetra-7. I) Tetra-8 J) Arixtra. K) Hexa.	147
Figure 4.3. [¹ H, ¹⁵ N] HSQC spectra of a) porcine intestinal mucosa heparin, b) porcine intestinal mucosa heparin treated with heparinase-III, c) 2- <i>O</i> -desulfated porcine intestinal mucosa heparin. All spectra were measured at 20° C.	152
Figure 4.4. [¹ H, ¹⁵ N] HSQC spectra of enoxaparin measured as a function of pH. Sample pH is noted in each spectrum. Spectra were measured at 20° C.	153
Figure 4.5. 2D [¹ H, ¹⁵ N] HSQC-TOCSY spectrum of enoxaparin measured at pH 8.2 at 20° C. Sulfamate NH correlations are labeled with Roman numerals.	156
Figure 5.1. SEC profiles of LMWHs A) tinzaparin, B) enoxaparin. The abbreviation dp signifies depolymerization product indicating the number of monosaccharides per chain.	168
Figure 5.2. Illustration of the RPIP separation mechanisms involving the charged analyte (here a heparin disaccharide), IPR and C18 stationary phase; A) dynamic coating of a C18 column with the IPR which separates analytes via an ion-exchange mechanism, B) formation of an ion-pair in the mobile phase which migrates to the stationary phase.	169
Figure 5.3. RPIP-UPLC-MS chromatograms measured for tinzaparin using 10 mM DBA and MeOH as the organic mobile phase with analysis times of (A) 40 min and (B) 100 min.	177

Figure 5.4.	179
Identical separation conditions for the separation of tinzaparin where IPR used in A) TrBA, B) DBA, and C) PTA.	
Figure 5.5.	181
Optimized 40 min RPIP-UPLC-MS separations of tinzaparin using ACN as the organic mobile phase and 10 mM A) DBA, B) TrBA, and C) PTA in both the aqueous and organic phases.	
Figure 5.6.	184
RPIP-UPLC-MS separation of tinzaparin using 10 mM PTA and ACN as the organic mobile phase with analysis times of A) 20 min and B) 100 min.	
Figure 5.7.	188
Mass spectra corresponding to chromatographic peaks from the optimized 40 min separation using PTA and ACN assigned as A) $\Delta 4,6,0$ and B) $\Delta 8,12,0$, and C) $\Delta 16,24,0$.	
Figure 5.8.	191
Extracted ion chromatogram for the mass of 913.29 ± 0.5 Da corresponding to the m/z of the singly charged oligosaccharide with configuration $\Delta 4,3,0$.	
Figure 5.9.	193
Chromatographic fingerprints of (A) tinzaparin and (B) enoxaparin with peak annotations. The separation conditions are identical to those presented in Figure 5.5C.	
Figure 5.10.	195
Analysis of enoxaparin using PTA and ACN. (A) Expansion of the total ion chromatogram shown in Figure 5.9B to better show the peaks of the lower molecular weight oligosaccharides. (B) Extracted ion chromatogram for mass 576.80 ± 0.5 Da corresponding to the m/z of the singly charged oligosaccharide with configuration $\Delta 4,6,0$	
Figure 5.11.	197
Total ion chromatograms for separations using PTA and ACN of (A) Clexane, (B) U.S.P. enoxaparin, and (C) E.D.Q.M. enoxaparin. The asterisk in (A) denotes the $\Delta 6,9,0$ peak and in (B) X identifies unique peaks not observed in the other chromatograms. The numbered peaks in chromatogram A are quantitatively compared in Figure 5.12.	

Figure 5.12. 198

Intensities of selected chromatographic peaks as labeled in Figure 5.11. Samples were measured in triplicate and plotted error represents 2 times the standard deviation. Peak intensities are normalized to the most intense peak ($\Delta 6,9,0$).

LIST OF TABLES

Table 2.1. 76

Summary of the ^1H and ^{15}N NMR chemical shifts (in ppm) measured using the [^1H - ^{15}N] HSQC-TOCSY pulse sequence for the compounds studied. Chemical shifts measured using one-dimensional ^1H NMR spectra are reported to three decimal places. Chemical shifts determined from 2D spectra are reported to two decimal places due to the poorer digital resolution of these measurements. Asterisks denote peaks where resonance overlap limits peak resolution.

Table 2.2. 87

Summary of the ^1H and ^{15}N NMR chemical shifts (in ppm) measured using the [^1H , ^{15}N] IMPACT-HNMBBC experiment for the compounds studied. Chemical shifts measured using one-dimensional ^1H NMR spectra are reported to three decimal places. Chemical shifts determined from 2D spectra are reported to two decimal places due to the poorer digital resolution of these measurements.

Table 3.1. 114

GlcNS NH temperature coefficients measured in 90% H_2O /10% D_2O 20 mM phosphate buffer.

Table 3.2. 120

Activation energies for the chemical exchange of the GlcNS sulfamate protons with water. Errors were calculated through a numerical estimate of the covariance matrix and correspond to ± 2.48 times the standard error for each parameter in this two parameter fit. To compensate for systematic errors, the calculated errors for each measurement were rounded up to ± 0.1 kcal/mol.

Table 4.1. 145

Chemical shifts determined from the 600 MHz [^1H , ^{15}N] HSQC spectra shown in Figure 4.1.

Table 4.2.	146
Summary of peak assignments and ^1H and ^{15}N chemical shifts determined for a series of oligosaccharide standards. Residue number is counted from the non-reducing end. Spectra were acquired at 20 °C for all compounds except Hexa, which was measured at 17 °C.	
Table 4.3.	159
Summary of sulfamate peak assignments for the [^1H , ^{15}N] HSQC spectra presented in Figure 4.1.	
Table 5.1.	175
Gradient profiles used for each experiment.	
Table 5.2.	187
Average retention times and total ion counts with percent relative standard deviations (RSDs) for 5 separate Tinzaparin separations using the 40 min PTA and ACN method shown in Figure 5.	

CHAPTER ONE

Introduction

The research presented in this dissertation focuses on the development of new analytical tools for the characterization of anionic biopolymers. Particular emphasis is placed on the analysis of the heterogeneous polysaccharides heparin and heparan sulfate (HS). Analysis of heparin and HS remains a challenge due to their structural complexity. While other biopolymers such as proteins and nucleic acids are synthesized through a template-driven process, the regulatory control of the biosynthesis of heparin and HS is not as straightforward. These molecules are initially synthesized as the homogenous precursor *N*-acetylheparosan which is acted upon by a series of enzymes that introduce heterogeneity through deacetylation, *N*- and *O*- sulfation, and uronic acid epimerization at various points throughout the polymer chain. Due to this inherent microheterogeneity, the bottom-up approach is typically taken in the characterization of these materials. This involves the size and charge fractionation to facilitate the isolation of individual oligosaccharides, followed by their identification using nuclear magnetic resonance (NMR) and mass-spectrometric analysis.

This dissertation builds on work performed by previous group members John Limtiaco, in the application of ^{15}N NMR for characterization of glycosaminoglycans, and Christopher Jones, in the separation of heparin-derived oligosaccharides by reversed-phase ion-pairing – liquid chromatography (RPIP-LC). The objectives of this dissertation are outlined below.

Objective 1: Explore the solution conditions that facilitate detection of the biologically important sulfamate (NHSO_3^-) groups of *N*-sulfoglucosamine (GlcNS) in heparin and HS by ^1H NMR and use these conditions to adapt existing methods for indirect detection of ^{15}N resonances in GlcNS (Chapter 2).

Objective 2: Explore the ability of the sulfamate ^1H NMR resonances to report on hydrogen bonds through measurements of the kinetics of exchange of the nitrogen bound protons with the aqueous solvent (Chapter 3).

Objective 3: Probe the local environment of GlcNS residues in unfractionated heparin, LMWH and HS using ^1H and ^{15}N chemical shift measurements for isolated oligosaccharides and chemically modified heparins in conjunction with differences in relative NH exchange rates (Chapter 4).

Objective 4: Explore the application of reversed-phase ion-pairing – ultraperformance liquid chromatography - mass spectrometry (RPIP-UPLC-MS) for fingerprinting and quality assurance of LMWHs (Chapter 5).

1.1. Glycosaminoglycans

Glycosaminoglycans (GAGs) are a class of linear highly anionic polysaccharides. They are comprised of repeating disaccharide units and are naturally polydisperse.

Members of this class include chondroitin sulfate (CS), dermatan sulfate (DS), heparin, heparan sulfate (HS), hyaluronan (HA) and keratan sulfate (KS). Generalized structures of the most common GAGs are shown in Figure 1.1.

GAGs are comprised of repeating disaccharide units composed of a hexose residue coupled to a hexosamine. The hexose can be an uronic acid (iduronic acid (IdoA) or glucuronic acid (GlcA)) or a galactose (Gal) residue. The hexosamine can be either glucosamine (GlcN) or galactosamine (GalN). HA is the only GAG that is not biosynthesized as a proteoglycan and is structurally the least diverse member of this family. The repeating disaccharide sequence of HA is $[-\text{GlcA}-\beta(1\rightarrow3)-\text{GlcNAc}-\beta(1\rightarrow4)]$ where GlcNAc represents *N*-acetylglucosamine. CS is comprised of $[-\text{GlcA}-\beta(1\rightarrow3)-\text{GalNAc}-\beta(1\rightarrow4)]$ disaccharide subunits which can be sulfated at the 2-*O* position of GlcA as well as the 4-*O* and 6-*O* positions of GalNAc. DS differs from CS by the epimerization of the uronic acid, which is exclusively IdoA in DS. KS is composed of $[-\text{Gal}-\beta(1\rightarrow4)-\text{GlcNAc}-\beta(1\rightarrow3)]$ with 6-*O*-sulfation possible for the Gal and GlcNAc residues.¹ Heparin and HS are the most structurally diverse GAGs and are comprised of $[-\text{UA}-\beta(1\rightarrow4)-\text{GlcN}-\beta(1\rightarrow4)]$ disaccharides in which the uronic acid can be either IdoA or GlcA and may be 2-*O*-sulfated.² The GlcN can be sulfated at the 3-*O* and 6-*O* positions and can be *N*-acetylated (GlcNAc), *N*-sulfated (GlcNS) or more rarely present as an unsubstituted primary amine.² Example structures can be found in Figure 1.1.

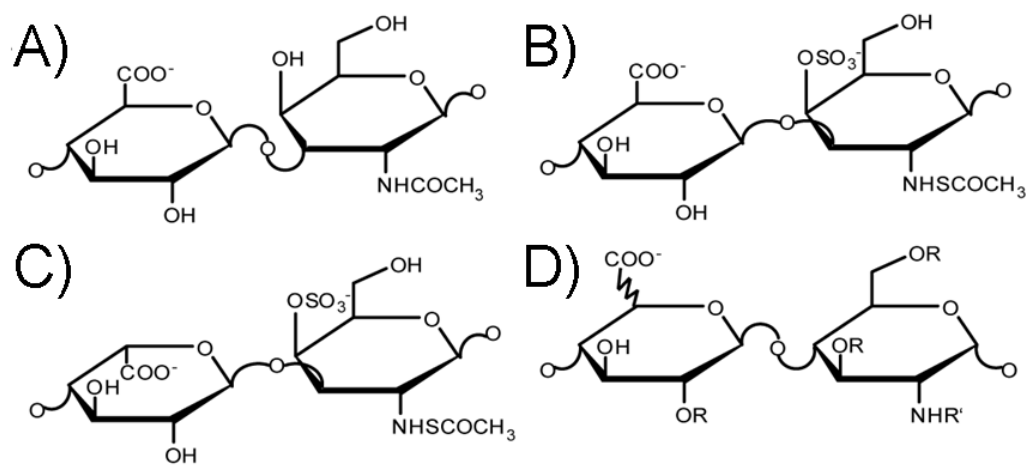


Figure 1.1. Haworth projections of GAG structures. A) HA, B) CS, C) DS, D) HS and heparin, where R= H or SO_3^- and R'= H, SO_3^- or COCH_3 .

1.2. Heparin and heparan sulfate

As illustrated in Figure 1.2, heparin is biosynthesized in a multistep process involving several enzymes in the endoplasmic reticulum and the Golgi apparatus of the mast cells of connective tissues. The homogeneous precursor, *N*-acetylheparosan is acted upon by a series of enzymes including *N*-deacetylase/*N*-sulfotransferase, 2-*O*, 3-*O*, and 6-*O* sulfotransferases and C5 epimerase to produce the microheterogeneous heparin biopolymer.² Although this process appears to occur in a specific and orderly manner, its regulation is not yet well understood. The structurally related HS proteoglycans are expressed, undergo enzymatic modifications similar to those described above, and are secreted by mammalian cells and strategically located on cell surfaces and in the extracellular matrix.^{3, 4}

Heparin is highly sulfated containing on average 1.8 - 2.6 sulfate groups per disaccharide⁵ with the most abundant disaccharide subunit being the trisulfated L-IdoA(2S)-D-GlcNS(6S). Although HS contains fewer sulfate groups per disaccharide (0.8 - 1.8)⁵ its structure has greater variability with regions of high and low sulfation and a greater percentage of GlcNAc and GlcA residues.

1.3. Biological significance

Heparin and HS influence numerous physiological⁶ and pathophysiological processes,⁷ including organo-,⁸ morpho-,⁹ angio-,¹⁰ and tumorigenesis;¹¹ growth control;¹² cell adhesion;¹³ inflammation;¹⁴ neural development and regeneration;^{15, 16} and hemostasis.¹⁷ Cell-surface HS proteoglycans also act as adhesion receptors for many viral

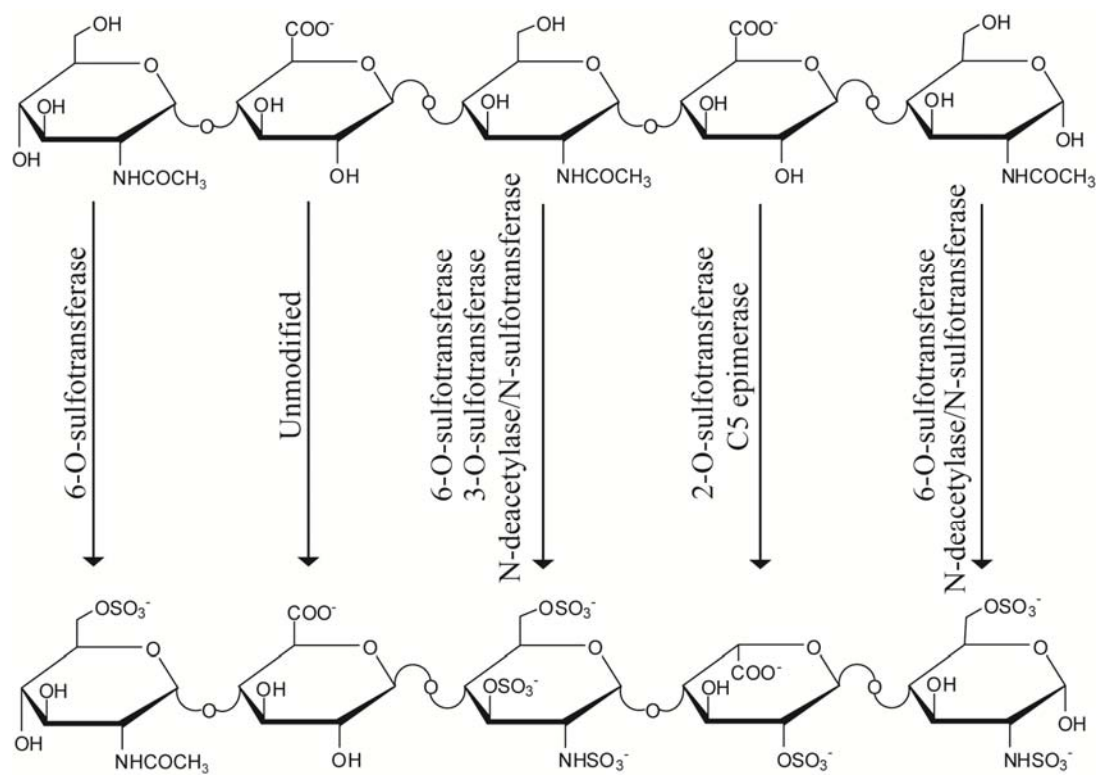


Figure 1.2. An illustration of the biosynthetic process converting the precursor *N*-acetylheparosan into heparin.

and bacterial pathogens, concentrating them on cell surfaces and increasing the pathogen's ability to bind to secondary receptors responsible for internalization.¹⁸⁻²⁰

Heparin is one of the oldest drugs in widespread clinical use. It is also one of the few currently used pharmaceutical agents derived from animal sources (namely porcine intestine).

Heparin mediates its biological functions through electrostatic interactions with basic amino acid residues of target proteins.²¹ The anticoagulant activity of heparin arises primarily through its interactions with the serine protease inhibitor antithrombin III. Binding of a specific heparin pentasaccharide sequence initiates a conformational change in antithrombin III that increases the flexibility of its reactive site loop and, as a result, its binding affinity for thrombin and other coagulation-cascade proteases. This pentasaccharide sequence is depicted as the biosynthetic product in Figure 1.2 and a synthetic pentasaccharide based on this sequence is commercially available and sold under the name Arixtra (Fondaparinux sodium). The structure of Arixtra is shown in Figure 1.3. Heparin is widely used in hemodialysis and in the initial treatment of venous thrombosis, pulmonary embolism, and acute coronary syndrome. A major drawback of heparin administration resides in the low predictability of coagulation parameters and the inherent risk of potential bleeding. To address these risks and limitations, low-molecular-weight heparins (LMWHs) were introduced into clinical use.²² LMWH are prepared from intact heparins through partial depolymerization and possess fewer side effects and an increased bioavailability. LMWHs are discussed in greater detail in Chapters 4 and 5.

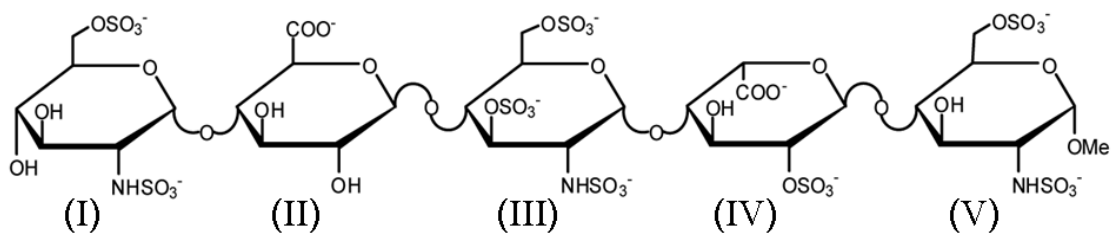


Figure 1.3. Antithrombotic heparin mimetic drug Arixtra with Roman numeral designations for each monosaccharide.

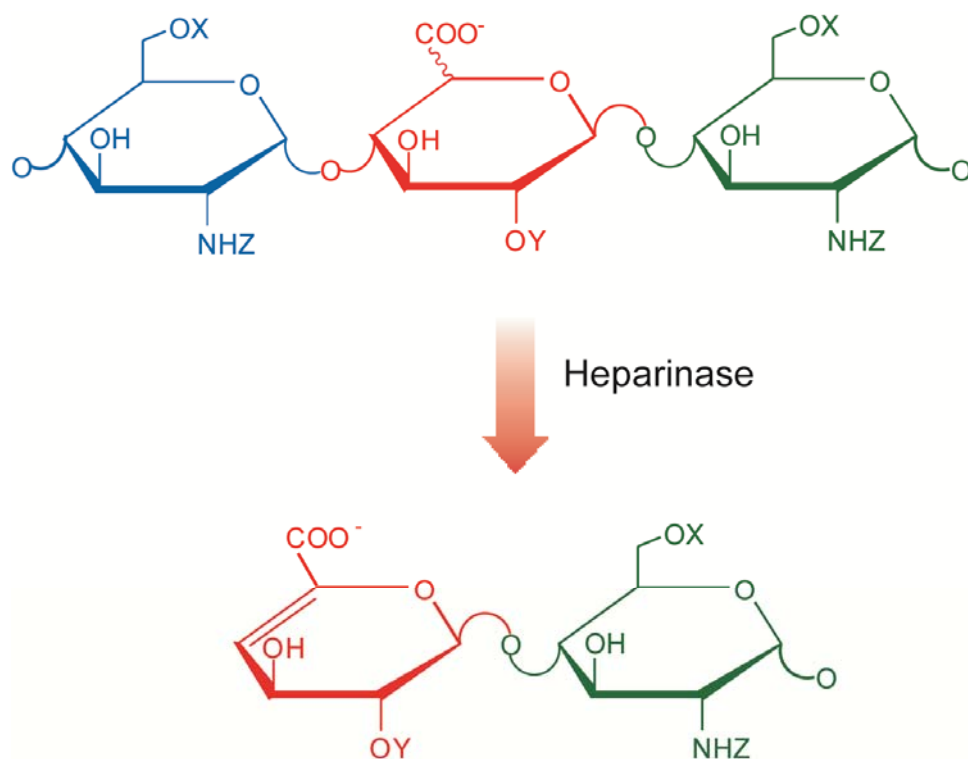
1.4. Depolymerization strategies.

Due to the high degree of structural diversity of heparin and HS, many methods used for their characterization utilize a bottom-up approach, whereby the intact polysaccharide chains are chemically or enzymatically depolymerized to smaller oligosaccharides prior to analysis.²³ In an exhaustive digestion, heparin and HS are reduced to their disaccharide building blocks facilitating their subsequent compositional analysis. LMWHs and larger oligosaccharides, which are more biologically relevant for use in protein binding studies, are obtained through partial depolymerization. Depolymerization techniques can be divided into two categories enzymatic and chemical, which are discussed in detail in the following sections.

1.4.1. Enzymatic depolymerization. Exhaustive digestions of heparin and HS are typically carried out by use of a cocktail containing the enzymes heparinase I, II, and III to selectively cleave the biopolymer at glucosamine (1→4) uronic acid glycosidic bonds. The enzymatic reaction inserts a double bond at the non-reducing end of each cleaved chain to create an ultraviolet (UV) chromophore that absorbs at a wavelength of 232 nm (e.g., Figure 1.4), thereby facilitating detection.²⁴ Such heparin lyase enzymes, produced by *Flavobacterium heparinum*, are highly specific to heparin and HS and are classified according to their substrate specificity.²⁵ The various heparinase specificities and the structures of the resulting cleavage products are illustrated in Figure 1.4. Heparinase I cleaves the polymer chain between GlcNS and 2-O-sulfated IdoA residues—the most common substitution motif in most forms of intact heparin. Heparinase II is less specific; it cleaves between glucosamine residues that can be *N*-sulfated or *N*-acetylated and 2-O-

sulfated IdoA, unsubstituted IdoA, or GlcA residues.²⁵ Heparinase III cleaves specifically at sites between *N*-acetylated or *N*-sulfated glucosamine and 2-*O*-unsubstituted IdoA or GlcA. Because the disaccharide GlcNAc(1→4)GlcA is commonly found in HS, heparinase III is often used for HS digestions.

1.4.2. Chemical depolymerization. Chemical depolymerization of heparin and HS oligosaccharides can occur by several means: β -elimination, reductive deamination, and oxidation. The specificities and reaction products are shown in Figure 1.5. β -Elimination mimics enzymatic cleavage through a chemical reaction that introduces a double bond at the non-reducing end of each cleaved oligosaccharide. The depolymerization is carried out through a two-step reaction in which the carboxylate group on the C5 carbon of the non-reducing-end hexuronic acid is reacted with a benzyl halide to form an ester. Then, a strong base extracts the proton at the C5 position of the non-reducing-end uronic acid, resulting in the formation of a double bond between C4 and C5. The benzyl ester is then eliminated through hydrolysis in basic solution.²⁶ In addition to the unsaturated uronic acid residue at the non-reducing end, the process of chemical β -elimination results in the formation of 1,6 anhydro structures at the reducing end GlcN residues as well as epimerization of reducing end GlcN residues to mannosamine (Man).²⁷



Heparinase I ^a	Heparinase II ^b	Heparinase III ^b
X = SO ₃ ⁻ or H	X = SO ₃ ⁻ or H	X = SO ₃ ⁻ or H
Y = SO ₃ ⁻	Y = SO ₃ ⁻ or H	Y = H
Z = SO ₃ ⁻	Z = SO ₃ ⁻ or H	Z = SO ₃ ⁻ or Ac
^a Hexuronic acid must be IdoA		
^b Hexuronic acid can be IdoA or GlcA		

Figure 1.4. Illustration of heparin lyase I, II, and III depolymerization products and substrate specificity.

Reductive deamination is typically performed with nitrous acid and the specificity of cleavage is determined by the solution pH. If the pH is at or below 1.5, cleavage occurs at GlcNS residues, whereas if the pH is 4.0, cleavage occurs at *N*-unsubstituted GlcN. To cleave GlcNAc glycosidic bonds via reductive deamination, samples need to first be deacetylated and then treated with nitrous acid at pH 4.0. Reductive deamination alters the structure of the glucosamine, producing a 2,5-anhydro-D-mannose residue at the reducing end of the cleaved oligosaccharide.²⁸ Oxidative depolymerization is performed with a combination of hydrogen peroxide and divalent copper or iron ions.²⁹ This reaction requires vicinal diols at the C2 and C3 positions of the hexuronic acid residue and results in cleavage and subsequent formation of an oxidized fragment of the hexuronic acid.³⁰

1.5. Separation of Oligosaccharides

Because heparin depolymerization reactions produce a complex mixture of variously substituted oligosaccharides, it is usually necessary to incorporate one or more separation steps into the analysis. If the goal of an experiment is characterization of the disaccharide composition of a heparin sample, an exhaustive digestion must be used to reduce the biopolymer to its component disaccharides. Although direct analysis of heparin digests can be performed with MS,³¹⁻³⁴ most studies perform compositional analysis with a separation method such as high-performance liquid chromatography (HPLC) to resolve the individual oligosaccharides. For samples that are depolymerized through enzymatic or β -elimination reactions, compositional analysis has been simplified

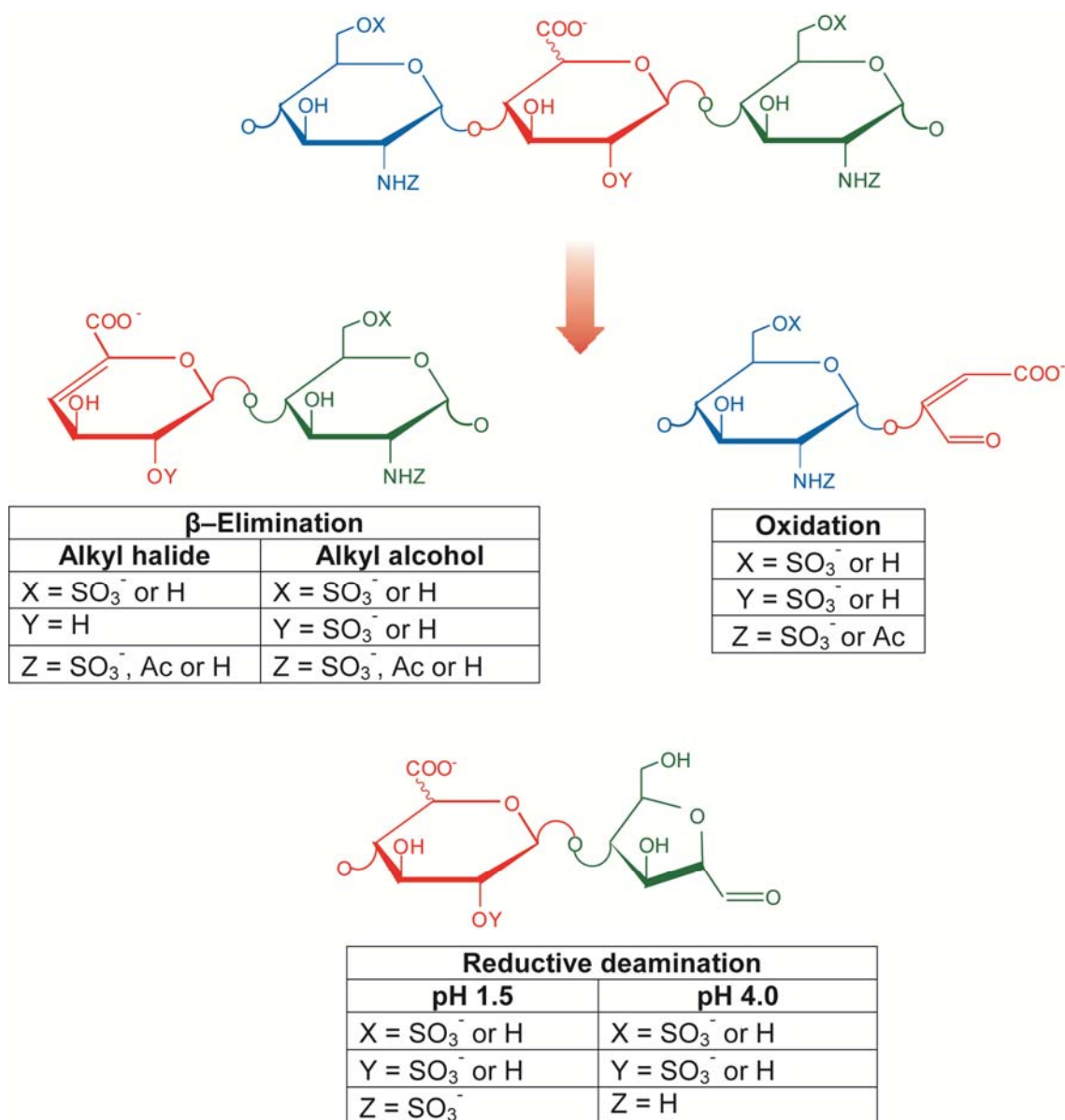


Figure 1.5. Illustration of chemical depolymerization products and reaction specificities.

by the commercial availability of authentic disaccharide standards. Frequently, the experimental goal is isolation and characterization of larger heparin oligosaccharides, often as part of a study to explore their protein-binding properties or biological activity. In such cases, the digestion is quenched before all the heparin is reduced to its component disaccharides. The analysis of the resulting mixture is complicated by the presence of variously sized oligosaccharides consisting of a diverse set of positional and configurational isomers. For such samples, a single separation cannot adequately resolve the individual components, and two or more orthogonal separation approaches may be employed.

1.5.1. Size-exclusion chromatography. Size-exclusion chromatography (SEC) is generally the first step in the analysis of a partially depolymerized heparin sample. This step resolves the mixture of heparin-derived oligosaccharides into size-uniform fractions.^{35, 36} Figure 1.6 shows the results of the preparative-scale SEC separation of a porcine intestinal mucosa heparin sample that was partially digested with heparinase I. This SEC separation, conducted with a Bio-Gel P10 fine gel packed into a 3 cm × 200 cm column, resolved the component oligosaccharides up to the hexadecasaccharide peak. Because of the specificity of the enzymatic reaction, primarily even-numbered oligomers are produced, although small quantities of trisaccharides have also been reported.³⁶ Preparative SEC can be carried out on a scale ranging from 100 mg to 1 g, which allows the resolution of size-uniform fractions for studies of heparin-protein interactions or for subsequent separation to yield purified single-component oligosaccharides. A common approach involves collection of the preparative SEC eluates in volumes of a few

milliliters to define each peak in the chromatogram. Upon completion of the separation, the collected fractions that correspond to a given oligomer size are pooled for subsequent separation according to charge. However, SEC peaks are not necessarily homogeneous, and the practice of pooling can make it more difficult to resolve trace components in the secondary separation.^{36, 37} Preparative-scale SEC separations generally take on the order of days to complete and, therefore, are not useful for the rapid characterization of heparin. In contrast, analytical SEC separations require only a few hours to achieve the same size fractionation using microgram quantities of material, which makes this method amenable to analysis of heparin and HS samples that are available only in limited amounts. These characteristics make analytical SEC a useful tool for rapid heparin analysis, especially when coupled directly to a secondary separation or a highly selective detection method such as MS.^{36, 38-40}

1.5.2. Ion-exchange chromatography. HPLC can offer a robust approach for the separation and analysis of heparin and HS oligosaccharides. Strong anion exchange (SAX)-HPLC is often used for the separation of GAG oligosaccharides, especially those derived from heparin and HS.^{35, 41-43} As with other separation techniques, analytical SAX columns provide the highest resolution, whereas preparative-scale columns have greater sample capacity. Preparative or semipreparative SAX separations allow the injection of larger quantities and the isolation of purified component oligosaccharides for subsequent characterization

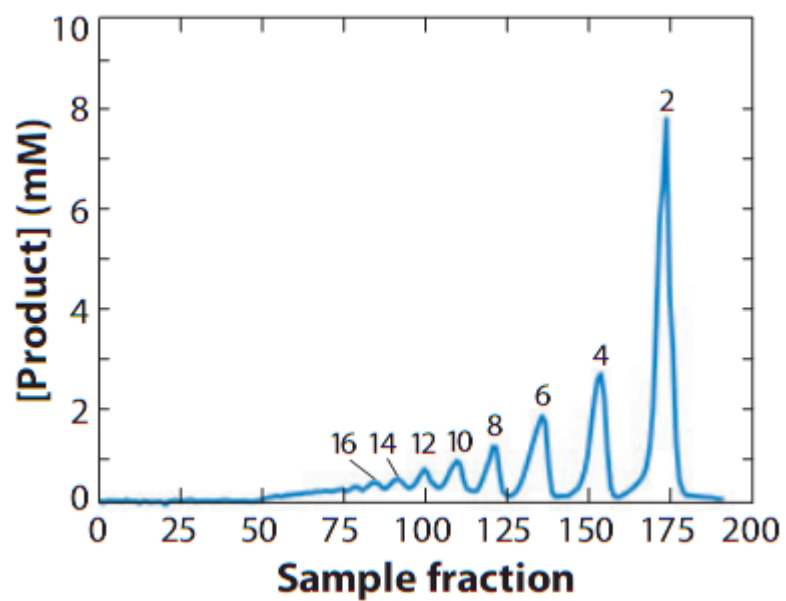


Figure 1.6. SEC chromatogram of 1g of heparinase-I digested porcine mucosal heparin separated on a 3 x 200 cm packed column. Numbers represent the number of monosaccharide residues comprising the depolymerization product. Product concentration was determined using the UV absorbance at 232 nm and a molar extinction coefficient of $5400 \text{ M}^{-1}\text{cm}^{-1}$.

experiments. Because of the complexity of heparin digest samples, a common protocol involves the injection of size-uniform SEC fractions onto the SAX column. The semipreparative SAX-HPLC separation of a heparin-derived SEC hexasaccharide fraction (Figure 1.7) shows excellent resolution between individual components. This resolution comes with a cost; the SAX separation requires much longer analysis times than either capillary electrophoresis (CE) or reversed-phase ion-pairing (RPIP) - HPLC (discussed in sections 1.6.3 and 1.6.4). Another disadvantage of SAX-HPLC is the high-ionic strength mobile phase (e.g., 2 M NaCl) required for the elution of highly charged heparin oligosaccharides. As a result, SAX is not easily amenable to detection by MS, and components isolated using this approach must be desalted prior to characterization by MS or NMR.

An alternative to SAX separations is weak anion exchange (WAX)-HPLC. The WAX column is packed with an amino-bonded stationary phase that interacts more weakly with anionic analytes than do the quaternary amine functionalized polymers used for SAX separations. As a result, WAX separations require less salt to elute highly charged analytes, making this method more compatible with hyphenated techniques such as HPLC-NMR. Limtiaco et al. showed that WAX could be used to separate DS, heparin, and the semisynthetic oversulfated chondroitin sulfate (OSCS) through a displacement-based mechanism.⁴⁴

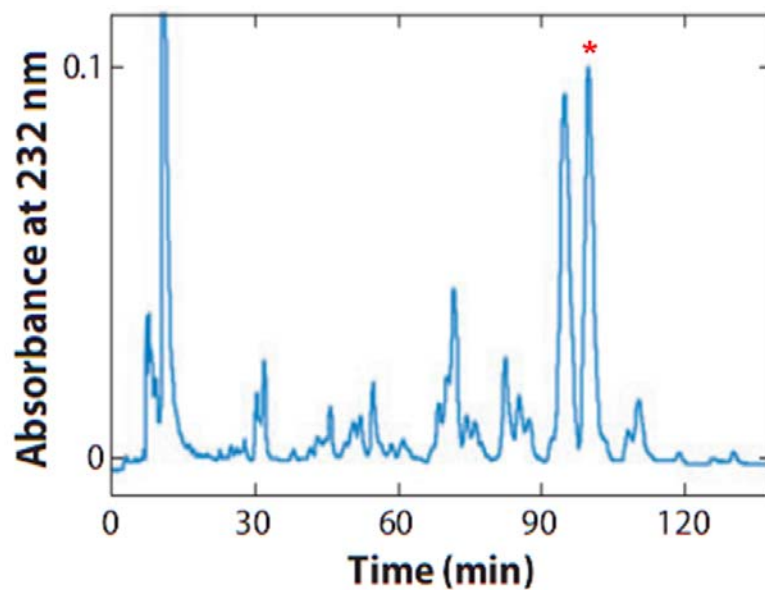


Figure 1.7. SAX chromatogram of hexasaccharide fraction (peak 6) of heparinase-I digested porcine mucosal heparin isolated from the SEC separation illustrated in Figure 1.6. The asterisk denotes hexasaccharide Δ UA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S), which is discussed in Chapter 4.

1.5.3. Reversed-phase ion-pair - ultraperformance liquid chromatography (RPIP-UPLC). RPIP-HPLC is an increasingly important method for the separation of heparin and HS oligosaccharides. The popularity of this approach stems from the widespread availability of reversed-phase HPLC columns and instruments as well as the ease of implementation of RPIP-HPLC with a variety of detection methods (e.g., UV, fluorescence, and MS). RPIP-HPLC is typically performed on octadecyl (C18) columns with a lipophilic alkyl ammonium salt used as an ion-pairing reagent (IPR). The IPR aids in the retention and resolution of the charged analyte through electrostatic interactions. The transient analyte-IPR ion pair is hydrophobic and the interaction with the IPR helps to neutralize the analyte charge facilitating interactions with the hydrophobic stationary phase of the reversed-phase HPLC column.⁴⁵⁻⁴⁹ Although the earliest separations used quaternary ammonium salts such as tetrabutylammonium, incorporation of more volatile reagents such as di-⁵⁰ and tributylamine^{45, 46} has made RPIP-HPLC more amenable to detection by MS. Hyphenation with MS provides important structural information about the analytes.⁵¹⁻⁵³

Smaller column-packing materials (e.g., 2 μm or less) can improve the speed and sensitivity of heparin analysis using RPIP-HPLC.^{45, 46, 52} Commercially available UPLC instruments utilize 1.7- μm -particle columns and proprietary mobile-phase pumps that can withstand the high pressures (up to 15,000 psi) needed to push mobile phase through the column. The speed of analysis and high resolution provided by RPIP-UPLC separations make this method an excellent choice for the analysis of heparin and HS. RPIP-UPLC separations facilitate the complete resolution of all possible heparin disaccharides,

including positional isomers.⁵³⁻⁵⁶ Separations of larger heparin-derived oligosaccharides have been reported as well.^{51, 52, 57-60} RPIP-UPLC has a distinct advantage in terms of the ease with which it can be coupled to MS detection when volatile IPRs are used. This advantage is especially significant given the lack of commercially available standards for heparin-derived oligosaccharides.

1.6. Mass spectrometry

MS is a useful and sensitive technique that can provide oligosaccharide molecular weight, the degree of sulfation and acetylation, monosaccharide composition, and under controlled experimental conditions, sequence.^{61, 62} Most MS studies of heparin and HS utilize electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI), although, the recently introduced method of electron detachment dissociation (EDD) shows great promise.

1.6.1. Negative-mode electrospray ionization mass spectrometry. Because of the highly anionic nature of heparin and HS, negative-mode ESI-MS is an attractive choice for their analysis.⁶³ Given the complexity of depolymerized heparin samples, another benefit of ESI-MS is the ease of hyphenation with separation techniques such as CE and HPLC. For example, in Chapter 5 RPIP-UPLC-ESI-MS is used to fingerprint the complex mixtures present in LWMH samples. Often, however, negative-ion ESI-MS spectra of heparin oligosaccharides are complicated by the presence of sodium and potassium adducts. Careful desalting, very clean sample preparation and instrumental components, and the use of ammonium buffers can reduce adduction.³⁴ An advantage of

coupling RPIP separations with MS detection is that salt adducts can be further reduced because sodium and potassium ions are largely eluted in the void volume of the column, whereas heparin oligosaccharides are retained through their interaction with the IPR.⁵⁵ Another challenge in the characterization of heparin and HS oligosaccharides by use of MS is the prevalence of sulfate loss during ionization. Sulfate loss from oligosaccharides may be catalyzed by trace amounts of acid; thus, one must be careful to remove acid from the analyte solution and source before performing MS analysis.⁶⁴ It is also important to choose ionization and ion-extraction conditions carefully, as sulfate loss can occur within the source region of the ESI interface.

1.6.2. Matrix assisted laser desorption ionization mass spectrometry. MALDI is a soft ionization method that enables relatively high throughput MS analysis on small quantities of sample. As in ESI-MS, a common problem encountered in the use of MALDI-MS for analysis of heparin oligosaccharides is fragmentation, especially sulfate loss, during the ionization process. Early work showed that complexation of oligosaccharides by synthetic (Arg-Gly)_n peptides or by the small basic protein angiogenin allowed the oligosaccharide to remain intact through the ionization process.⁶⁵ The full oligosaccharide-peptide complex was detected by positive-ion MS, and the molecular weight of the oligosaccharide was calculated by subtraction. Later studies used a similar complexation approach through the addition of quaternary ammonium or phosphonium salts as a co-matrix for MALDI-MS.⁶⁶ The use of cesium salts to suppress sulfate loss when using MALDI-MS in an ionic liquid matrix has also been explored.⁶⁷ Tissot et al. tested the ionic liquid 1-methylimidazolium α -cyano-4-hydroxycinnamate as

a MALDI matrix and successfully ionized heparin di-, tetra-, hexa-, octa-, and decasaccharides.⁶⁸ Dihydroxybenzoic acid and norharmane are also effective MALDI matrices for the structural characterization of heparin and HS by MS.⁶⁹ Clearly, MALDI-MS has great potential for the characterization of heparin and HS oligosaccharides.

1.6.3. Tandem mass spectrometry. The use of tandem MS (MS/MS) to determine both the structure and the sequence of oligosaccharides is an ongoing area of interest in GAG research. One limitation of this approach is the propensity of heparin oligosaccharides to fragment via sulfate loss instead of through more informative ring cleavages. Zaia & Costello demonstrated that the degree of fragmentation of any oligosaccharide is highly dependent upon the charge state of the ion selected for collision-induced dissociation (CID).⁶³ These authors showed that through careful selection of charge states and addition of calcium ions to stabilize the sulfate groups, the degree and abundance of ring cleavage ions can be enhanced. Meissen et al. reported that MS/MS with CID can be used to distinguish 3-O-sulfation sites from 6-O-sulfation sites in heparin oligosaccharides by inducing specific cross-ring cleavages.⁷⁰ Kailemia et al. reported the complete mass spectral characterization of the synthetic pentasaccharide Arixtra using collision-induced dissociation (CID) MS/MS.⁶² Ultimately, the ability to use tandem MS to sequence GAG oligosaccharides requires the generation of unique and informative fragments for each monosaccharide. In addition to cleavage of glycosidic linkages, this process also requires numerous cross-ring cleavages to distinguish variable points of sulfation and acetylation. MALDI-LIFT-TOF/TOF and ESI-CID-MS/MS were compared for the structural analysis of *N*-acetylheparosan (a biosynthetic heparin

precursor) oligosaccharides.⁷¹ This study showed that MALDI-LIFT-TOF/TOF yielded much better cleavage specificity than did CID. More recently, the advancement of the MS/MS technique EDD has proven useful for creating unique cross-ring cleavages in heparin and HS. By using EDD with Fourier transform ion cyclotron resonance, Wolff et al. showed that diagnostic fragment ions can be created that distinguish IdoA from GlcA in tetrasaccharides.⁷² Further advances in the use of EDD will probably lead to exciting new applications of MS/MS for the characterization of heparin and HS oligosaccharides.

1.7. Nuclear magnetic resonance spectroscopy

NMR spectroscopy is highly sensitive to minor variations in molecular structure, making it an important technique for heparin characterization. With the powerful arsenal of one- and two-dimensional experimental techniques available, NMR spectroscopy can be used to determine the sequence of the component monosaccharide residues and unambiguously determine sites of *N*-acetylation as well as of *N*- and *O*-sulfation along the oligosaccharide chain. Most importantly, NMR spectroscopy can also specify the orientation of the anomeric linkage connecting the various disaccharide subunits and easily distinguishes IdoA and GlcA epimers. Because of its high level of microheterogeneity and polydispersity, the complete structural characterization of unfractionated heparin continues to be a major challenge. Interpretation of NMR spectral data is usually facilitated by molecular-weight and fragmentation information provided by MS. Several factors must be considered to ensure that satisfactory NMR spectra are acquired for heparin structural studies. For oligosaccharide analysis, components should

have a purity of greater than 80% to 90% to avoid complications in structural determinations. Although NMR is more salt tolerant than MS, high levels of salt in oligosaccharide samples isolated from SAX separations can make it impossible to properly tune and match the NMR probe, and a desalting step is usually required prior to analysis. As demonstrated by McIwen,⁷³ heparin resonance line widths are severely affected by binding of trace paramagnetic impurities. Paramagnetic transition-metal ions, which may be present as production impurities or introduced during oligosaccharide isolation, can cause line broadening through paramagnetic relaxation enhancement. The addition of a small amount of deuterated EDTA(ethylenediaminetetraacetic acid) significantly improves the spectral quality for both unfractionated heparin⁷³ and LMWH samples, and it can also be helpful in the analysis of isolated oligosaccharides.⁷⁴

1.7.1. ¹H NMR spectroscopy analysis of glycosaminoglycans. The ¹H nucleus has a high gyromagnetic ratio, natural abundance and a spin of ½, making it ideal for biomolecular NMR analysis. The starting point in the NMR analysis of GAGs is typically the acquisition of the one-dimensional ¹H spectrum, as shown in Figure 1.8. The ¹H NMR spectra of carbohydrates have several characteristic traits that can quickly provide useful information. The region from 1.8 - 2.1 ppm contains the characteristic singlet resonances of *N*-acetyl groups or reducing end methyl groups which may be introduced synthetically. Other resonances in this region can signify contaminants, for example for acetic acid, or residual components arising from the manufacturing process. For example, Sasaki et al. identified tris(2-n-butoxyethyl) phosphate as a low molecular weight contaminate in heparin which gives rise to resonances in this region.⁷

The region of the spectrum from 2.8 to 4.6 ppm contains most of the resonances of the sugar rings and exhibits a high degree of overlap. Due to insufficient dispersion in this region it can be difficult to extract structural information directly from the ^1H spectrum, especially for a complex sample such as a LMWH. The region from 4.6 to 6.0 ppm contains the resonances of the anomeric protons and is less crowded and more information rich. This region also contains resonances of the H4 protons of the unsaturated non-reducing end resulting from β -elimination cleavage as well as resonances of the exchangeable protons of sulfamate (NHSO_3^-) and hydroxyl groups. Because the sulfamate and hydroxyl protons exchange with the protons of the aqueous solvent, special care is needed to detect these resonances. The region between 6.2 and 10 ppm contains few GAG resonances. If the spectrum is acquired in H_2O , the exchangeable amide resonances of *N*-acetylated glucosamine or galactosamine residues will be detected.

The ^1H NMR spectra of HA, CS, DS, HS, heparin, enoxaparin, and Arixtra, are presented in Figure 1.8. Broader resonances are observed in the GAG spectra (Figures 1.8A-G) due to their greater average molecular weights. These spectra also reflect the relative heterogeneity of the GAG polymers ranging from the more uniform HA (Figure 1.8A) to the most complex enoxaparin (Figure 1.8F). General structural characteristics like acetylation and unsaturation at the non-reducing end are easily distinguished, but individual saccharide identification and connectivity is not readily extracted from the ^1H spectrum alone. The ^1H spectrum of Arixtra (Figure 1.8G), a pure pentasaccharide, contains many discrete resonances and peak multiplicities are easily observed. Still the

limited ^1H chemical shift dispersion hinders the ability to make detailed assignments and definitive structural characterization requires two-dimensional NMR spectra.

1.7.2. Homonuclear two-dimensional NMR spectroscopy. Two-dimensional (2D) NMR is a powerful tool for assigning the structures of heparin-derived oligosaccharides.⁷⁵ Homonuclear experiments that provide information about scalar or dipolar coupling are critical for teasing out information needed to make complete structural assignments of oligosaccharides. The primary NMR experiments for determining heparin-derived oligosaccharide structure are the total correlation spectroscopy (TOCSY) and correlation spectroscopy (COSY) pulse sequences and experiments that utilize dipolar interactions through the Nuclear Overhauser Effect (NOE). The strategy for resonance assignment using these homonuclear 2D NMR experiments is illustrated in this chapter using the synthetic pentasaccharide Arixtra.

The TOCSY spectrum provides an excellent starting point for oligosaccharide structural analysis. Similar to the individual amino acids in a peptide, the monosaccharide units in an oligosaccharide are isolated spin systems bracketed by glycosidic bonds. Because TOCSY relays chemical shift information throughout a spin system, TOCSY cross peaks to the well-resolved anomeric resonances can be used to measure the chemical shifts of all the protons within each Arixtra monosaccharide (Figure 1.9).⁷⁶ The critical TOCSY parameters are the mixing time and spin lock pulse length.

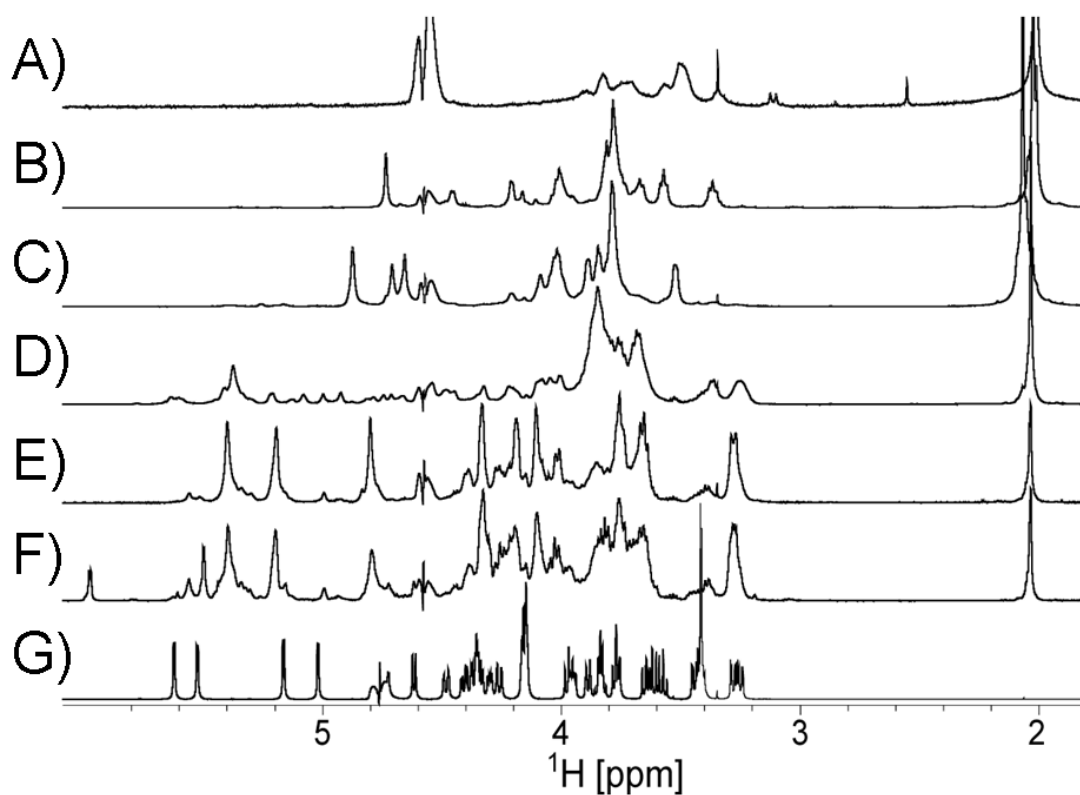


Figure 1.8. ^1H NMR spectra in order of increasing structural complexity (A-F), (A) hyaluronic acid (B) chondroitin sulfate, (C) dermatan sulfate, (D) heparan sulfate, (E) unfractionated heparin, (F) enoxaparin. (G) the pure oligosaccharide Arixtra. The structure of the Arixtra pentasaccharide is shown in Figure 1.3.

In practice, a mixing time of 120 ms is sufficient to transfer magnetization from the anomeric proton of GlcN to the H6 protons, which correspond to the furthest separated protons in heparin. For 600 MHz experiments with GAGs or GAG-derived oligosaccharides, a 35 - 45 μ s pulse (at the appropriate calibrated power level) is typically used for the spin lock.

Interpreting the TOCSY spectrum starts by identifying each monosaccharide by recognizing the anomeric protons and then tracing linkages to the other sugar protons within each monosaccharide residue as illustrated in Figure 1.9. The projections along F1 show the anomeric protons of each of the five monosaccharides and the horizontal rows give the TOCSY cross peaks to the other protons of the sugar rings. The TOCSY spectrum measured in 90% H₂O/10%D₂O can also be used to assign the exchangeable amide, sulfamate and hydroxyl protons under conditions of slow exchange, providing that care is taken in the choice of solvent suppression methods. In our experience, Watergate-W5 and excitation sculpting provide the best results for TOCSY spectra measured for this purpose.

For larger oligosaccharides spectral overlap in the anomeric region becomes a problem. To address this issue, the TOCSY and ROESY pulse sequences have been enhanced by adding band-selective homonuclear decoupling (BASHD). Both single BASHD, with homonuclear decoupling in F₁, and double BASHD experiments with decoupling in F₁ and F₂ have been reported.^{77, 78} The BASHD pulse sequences collapse multiplet resonances into singlets to resolve peaks with very similar chemical shifts.

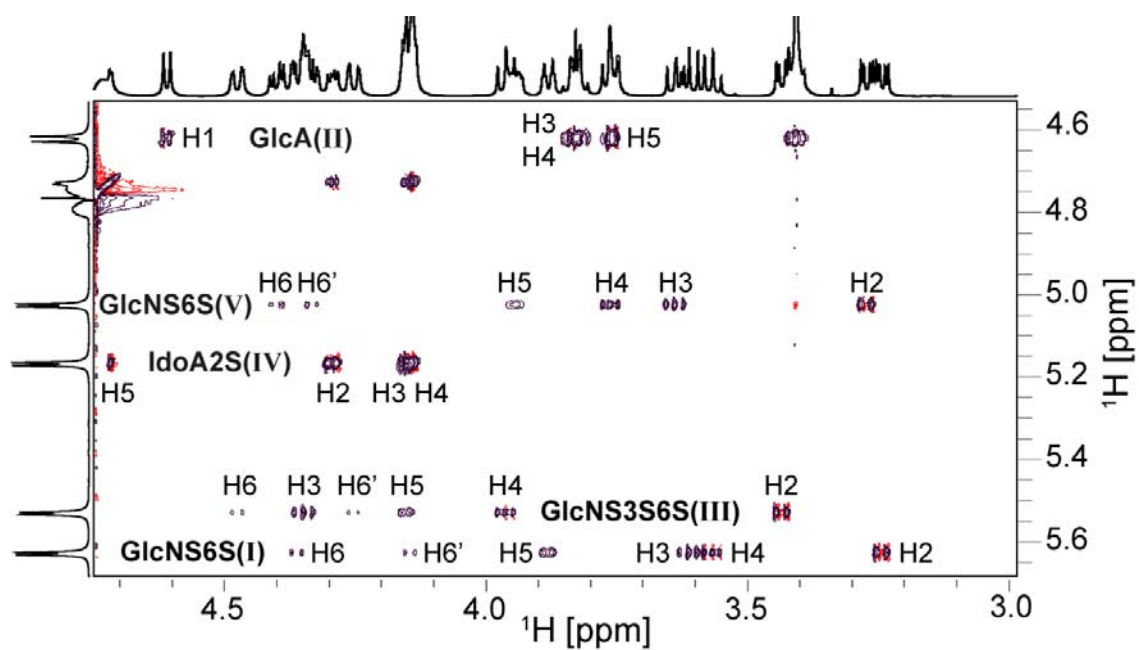


Figure 1.9. TOCSY spectrum measured for Arixtra in D_2O using presaturation with residue and proton assignments labeled.

Once the ^1H chemical shifts of each monosaccharide have been determined, the next step is to assign each peak to an individual proton of the sugar ring using the COSY cross peaks to connect the diagonal resonances of scalar coupled protons.⁷⁹ Preliminary interpretation of the TOCSY spectrum simplifies the COSY analysis, minimizing incorrect assignments. The double quantum filtered COSY (DQF-COSY) pulse sequence is particularly useful for oligosaccharide analysis.⁸⁰ The DQF-COSY is a phase-sensitive pulse sequence that produces peaks with pure absorption line shape while suppressing singlet resonances from the diagonal (Figure 1.10).⁸¹ Compared with the dispersive character of peaks obtained in absorption-mode experiments, the better resolution of the phase-sensitive DQF-COSY spectrum allows for the detection of cross peaks directly adjacent to the diagonal. This is especially important for assignment of H6 resonances in GlcN residues which are key for determining 6-O substitution. Overlap of the phase-sensitive COSY cross peaks can sometimes complicate interpretation. If coupling information is not required, the spectra can be processed in magnitude mode in F1 producing peaks with a discrete center that are more easily assigned (Figure 1.10B). When used with an appropriate solvent suppression method, such as excitation sculpting,⁸² or Watergate-W5,⁸³ the COSY experiment is the method of choice for assigning the exchangeable hydroxyl protons

Although the TOCSY and COSY spectra can be used to identify the component monosaccharides, complete structure elucidation requires determination of their sequence in the oligosaccharide, typically starting at the non-reducing end. Defining connections between monosaccharides requires spatial information determined from NMR

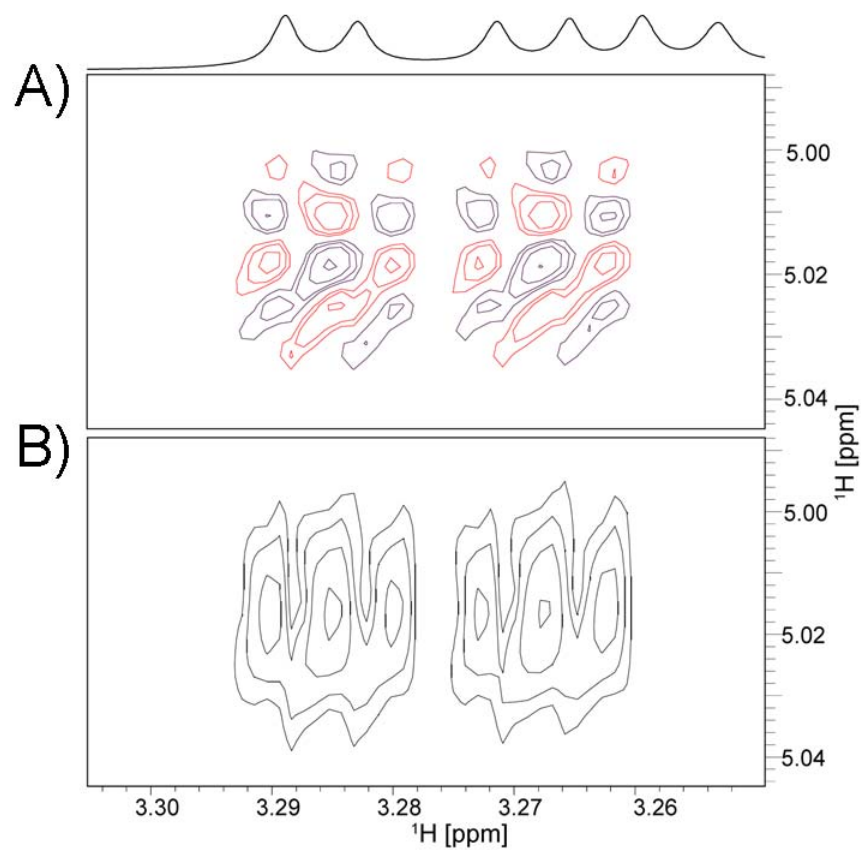


Figure 1.10. Expansion of the DQF-COSY spectrum of Arixtra showing the cross peak connecting H1 and H2 of the GlcNS6S(V) residue, A) processed in phase-sensitive mode in F2 and F1, B) processed in phase sensitive mode in F2 and magnitude mode in F1.

experiments that utilize the NOE. A common 2D NOE-based experiment is the nuclear Overhauser effect spectroscopy (NOESY) pulse sequence that detects through-space dipolar relaxation between protons within about 5 Å. NOEs are positive for small, rapidly tumbling molecules and negative for large molecules as reflected by the phase of the NOESY cross peaks relative to the diagonal. Between these motional extremes, the NOE passes through zero, which can pose a problem for detection of dipolar relaxation in medium-sized oligosaccharides.

The rotating frame Overhauser effect spectroscopy (ROESY) experiment provides an alternative approach to observing dipolar relaxation. The ROESY pulse sequence uses a spin lock during the mixing time to allow cross-relaxation in the rotating frame.⁸⁴ In the ROESY experiment all molecules (even large GAG biopolymers) give rise to positive ROE cross peaks that are opposite in sign to the diagonal. A disadvantage of the ROESY pulse sequence is that the spin lock can also cause coherence transfer between *J*-coupled spins producing COSY cross peaks. TOCSY cross peaks can also be generated through coherence transfer to *J*-coupled protons during the spin lock as a result of satisfying the Hartmann-Hahn conditions, especially when the spin lock is achieved by application of a long continuous wave pulse. COSY and TOCSY cross peaks can be easily distinguished from the desired ROESY cross peaks as they are opposite in sign. The T-ROESY pulse sequence generates the spin lock using a series of composite 180° pulses along the $\pm x$ axis to suppress TOCSY cross peaks by eliminating the Hartmann-Hahn match between coupled spins.⁸⁵

The ROESY spectrum of Arixtra (Figure 1.11) provides connectivities between the H1 and H4 protons across the glycosidic linkage. The cross-linkage correlations can be observed between Arixtra residues GlcNS6S(I)H1-GlcA(II)H4, GlcA(II)H1-GlcNS3S6S(III)H4, and GlcNS3S6S(III)H1-IdoA2S(IV)H4. While the correlation between residues IdoA2S(IV)H1-GlcNS6S(V)H4 is not observed, this cross peak is detected in the corresponding column in the F1 dimension. As in the acquisition of the TOCSY spectrum, the critical parameters for the ROESY experiment are the duration of the mixing time and the length and power of the spin lock pulses. A ROESY mixing time of 300 - 350 ms is sufficient for magnetization transfer across the oligosaccharide glycosidic bond. Because protons that are connected by spin-spin coupling are also close in space, overlaying the ROESY and TOCSY spectra can aid in the identification of inter-residue cross peaks that arise solely from cross-relaxation.

1.7.3. Heteronuclear two-dimensional NMR spectroscopy. The similar structures of the GAG disaccharide building blocks and the limited chemical shift dispersion of their ^1H NMR resonances makes characterization of even moderately-sized GAG oligosaccharides (e.g. decamers) difficult using only the homonuclear 2D NMR experiments described in section 1.8.2. Heteronuclear NMR experiments that correlate ^1H chemical shifts with those of ^{13}C or ^{15}N offer significantly improved spectral resolution and ^{13}C and ^{15}N chemical shifts have been used to assign the primary structures of GAG-derived oligosaccharides and to determine monosaccharide composition.^{86, 87}

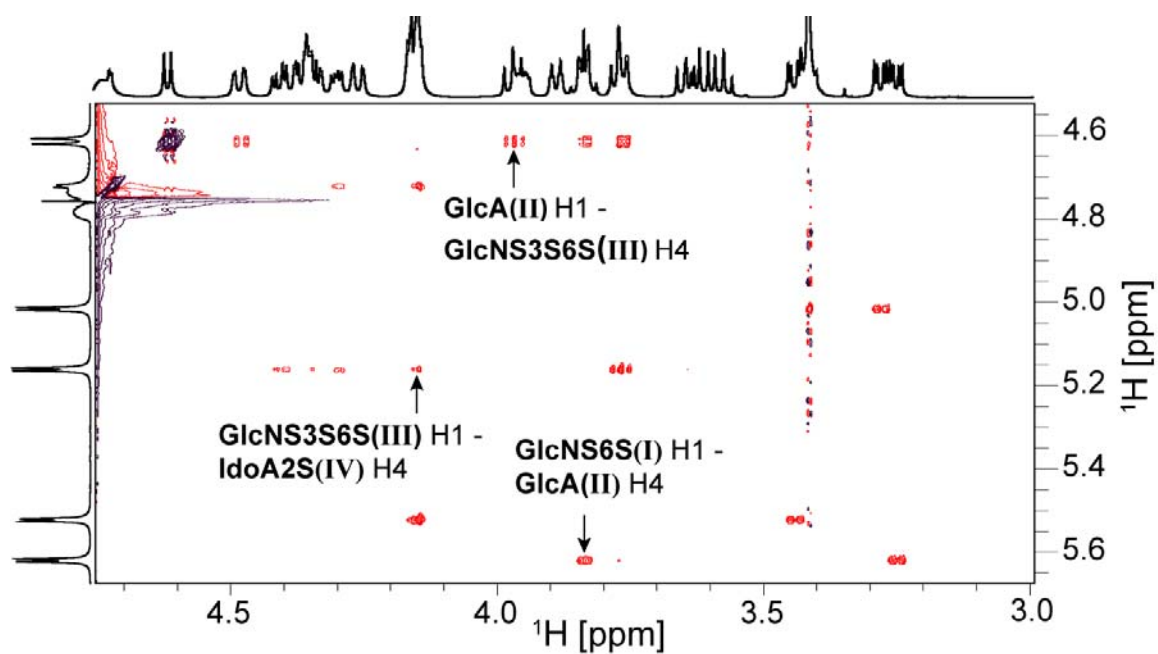


Figure 1.11 ROESY spectrum measured for Arixtra in D₂O using presaturation with through space correlations labeled.

The most commonly used heteronuclear NMR experiments in GAG structural studies are the heteronuclear single quantum coherence (HSQC) and the heteronuclear multiple bond coherence (HMBC) experiments.^{88, 89} The HSQC pulse sequence detects protons directly *J*-coupled to a heteronucleus while the HMBC experiment detects protons coupled to ¹³C or ¹⁵N through 3 or 4 bond couplings.

The indirect detection of heteronuclei through scalar-coupled protons using experiments like HSQC offers a significant sensitivity enhancement for nuclei with lower gyromagnetic ratios (γ) (e.g. ¹³C and ¹⁵N) compared to direct observation. This sensitivity enhancement is proportional to the ratio of the gyromagnetic ratios (γ) of the nuclei interrogated, e.g. $\gamma_{\text{high}}/\gamma_{\text{low}}$, where γ_{high} is that of ¹H and γ_{low} is that of the indirectly detected heteronucleus.⁸¹ This translates to an enhancement factor of about 4 for ¹³C and 10 for ¹⁵N compared to direct detection. In addition, because ¹H T_1 relaxation times are much shorter than those of ¹³C and ¹⁵N, saturation effects are reduced in indirect detection experiments giving a significant signal enhancement and allowing the use of short repetition times. Finally, inverse detection using the [¹H, ¹⁵N] HSQC experiment overcomes the negative NOE of ¹⁵N which can further reduce the signal in experiments that directly observe this nucleus.

Although many heteronuclear NMR experiments make use of ¹³C and ¹⁵N at natural abundance levels, 1.1% and 0.37% respectively, isotopic enrichment can greatly enhance the detection sensitivity. While it is not easy, it is possible to isotopically label some GAGs,⁹⁰ but because robust methods for overexpression of GAGs in bacteria (as is commonly done for the preparation of ¹³C and ¹⁵N labeled proteins) are still under

development, most samples are analyzed at natural abundance. Furthermore, measurements to ensure quality or examine protein binding of pharmaceutical samples of unfractionated heparin or LMWHs must be carried out with samples containing natural abundance levels of ^{13}C and ^{15}N .

Through the utilization of extensive chemical shift libraries, $[\text{}^1\text{H}, \text{}^{13}\text{C}]$ HSQC spectra are a powerful tool for GAG characterization. Work by Torri et al.⁹³ for heparin has shown that the characteristic ^1H and ^{13}C chemical shifts of the anomeric H1 and C1 resonances can be used to identify component monosaccharides. For example, the $[\text{}^1\text{H}, \text{}^{13}\text{C}]$ HSQC spectra measured for contaminated lots of pharmaceutical heparin were critical in the conclusive identification of OSCS as the impurity that led to the deaths of several hundreds of patients worldwide in 2008.^{91, 92} The $[\text{}^1\text{H}, \text{}^{13}\text{C}]$ HSQC experiment is particularly useful for the compositional analysis of oligosaccharide mixtures, like those found in LMWH heparin preparations. For example, Bisio et al. determined the monosaccharide composition of two LMWH heparin products tinzaparin and enoxaparin, which are manufactured through different processes.⁸⁷

Figure 1.12 presents the anomeric region of the $[\text{}^1\text{H}, \text{}^{13}\text{C}]$ HSQC spectrum of enoxaparin along with the assignments of the abundant correlations observed in this region. Extracting quantitative data from 2D NMR experiments can be challenging, however a strategy has been reported to accomplish this with good relative accuracy.⁹³ Focusing on the integration of anomeric (H1/C1) peaks for IdoA and GlcA residues and H2/C2 for GlcN residues and using an optimal $^1J_{(\text{CH})}$ of 155 Hz, as an average of the actual H1/C1 and H2/C2 coupling constants (139 Hz - 170 Hz) can account for small

differences in $^1J_{\text{(CH)}}$ and variability in T_2 relaxation times.^{87, 93} If knowledge of actual $^1J_{\text{(CH)}}$ values is required, they can be determined experimentally by acquiring a HSQC spectrum without heteronuclear decoupling during acquisition.

An alternative option for heteronuclear NMR analysis of GAGs is through the ^{15}N nuclei found in the glucosamine and galactosamine residues however this approach is underutilized compared with ^{13}C -based experiments. A challenge to observing ^{15}N chemical shifts through indirect detection experiments like HSQC is the chemical exchange of the nitrogen-bound protons with protic solvents. Chemical exchange precludes the use of D_2O as a solvent and, depending on the rate, exchange broadening can attenuate or even eliminate the NH resonances from the spectrum. Prior to the work described in this dissertation, the only NH groups analyzed by heteronuclear NMR in GAGs were those which are *N*-acetylated. Like the backbone amides of proteins, the GalNAc and GlcNAc amide protons exchange slowly with water due to the partial double bond character of the amide functional group. A benefit of studying *N*-acetylated galactosamine or glucosamine is that the amide ^1H resonances have chemical shifts between 7.9 and 8.5 ppm and are well separated from the solvent peak. As a result, most studies using ^{15}N chemical shifts to characterize GAG structures have focused on the *N*-acetyl moieties of HA,⁹⁴ CS,⁹⁵ and HS.⁹⁶⁻⁹⁸

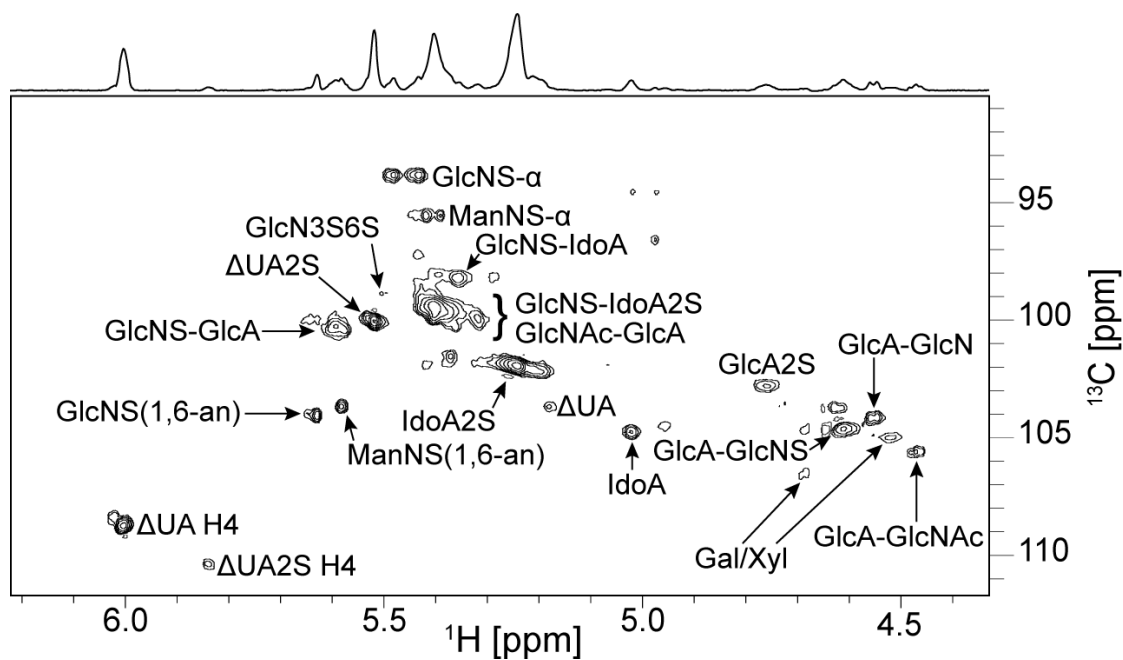


Figure 1.12. The anomeric region of the [^1H , ^{13}C] HSQC spectrum of enoxaparin with assignments labeled, where Man = mannose, and 1,6 an = 1,6 anhydro. Peaks labeled ΔUA reflect the unsaturated uronic acid resulting from depolymerization and those labeled Gal/Xyl are due to the galactose and xylose residues of the linking region.

1.8. Summary

The research presented in this dissertation is aimed at the development of analytical tools for the characterization of the biopolymers heparin and HS using both spectroscopic and chromatographic methods. Chapter 2 explores the solution conditions that facilitate detection of the ^1H and ^{15}N NMR chemical shifts of the sulfamate groups of the GlcNS residues found in heparin and HS. In Chapter 3 line shape analysis of the ^1H resonances of the sulfamate groups are used to probe elements of secondary structure of heparin-derived oligosaccharides in aqueous solution. Analysis of NH - solvent exchange kinetics led to our identification of the first solution state hydrogen bond between a sulfamate group proton and the 3-*O*-sulfate moiety of the heparin drug Arixtra (Fonduparinux sodium). In Chapter 4, ^1H and ^{15}N chemical shifts of GlcNS sulfamate groups were determined using a library of isolated and purified oligosaccharide standards and chemically modified heparins. This information was used to assign the correlations in [^1H , ^{15}N] HSQC spectra measured for unfractionated and LMW heparins as well as commercial and human derived HS samples. In this chapter we build on the structural hydrogen bond described in Chapter 3 to probe the pH dependence of HSQC cross peaks in spectra of enoxaparin and conclusively identify the 3-*O*-sulfated GlcNS correlations based on their reduced rate of solvent exchange. Chapter 5 shifts focus to the chromatographic separation and unique fingerprinting of LMWHs using the dynamic separation technique of RPIP-UPLC-MS. This chapter focuses on the comparison of chromatographic parameters as well as chromatographic and mass spectral assignments of components ranging in size from tetrasaccharides to a 16-residue oligomer.

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CHAPTER TWO

Detection and assignment of sulfamate ^1H and ^{15}N NMR resonances of *N*-sulfo-glucosamine

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Abstract: The glucosamine sulfamate (NHSO_3^-) groups are critically important structural elements of the glycosaminoglycans heparin and heparan sulfate. Experimental conditions are presented for detection of the sulfamate ^1H and ^{15}N NMR resonances in aqueous solution. NMR spectra reported for *N*-sulfoglucosamine (GlcNS) and the synthetic pentasaccharide drug Arixtra demonstrate the broad utility of the sulfamate group chemical shifts to reflect differences in molecular structure. The sulfamate protons also provide an efficient route for detection of ^{15}N chemical shifts through proton–

nitrogen correlations measured with the heteronuclear single quantum coherence (HSQC) experiment. The information provided by these single quantum correlations can be expanded using the [^1H , ^{15}N] HSQC-TOCSY and [^1H , ^{15}N] IMPACT-HNMBC experiments to correlate ^{15}N chemical shifts to the carbon-bound protons for the purpose of identifying and characterizing the constitutional aminosugars.

2.1 Introduction

NMR has proven to be an invaluable method for characterizing heparin isolated from biological sources,¹ as well as synthetic oligosaccharides,² and analogs produced from related materials, for example the *E. coli* K5 bacterial capsular polysaccharide heparosan.^{3,4} The value of NMR for GAG characterization was aptly demonstrated during the 2007-08 contamination of the pharmaceutical heparin supply chain with oversulfated chondroitin sulfate (OSCS).⁵ In the months following the initial clinical observations of anaphylaxis following heparin administration, NMR spectroscopy played a crucial role in determining the structure of the OSCS contaminant and in screening lots intended for pharmaceutical use.^{5,6}

Characterization of the intact heparin and heparan sulfate (HS) biopolymers is complicated by their inherent complexity due to both structural microheterogeneity and polydispersity.⁷ Despite this complexity, [^1H , ^{13}C] HSQC NMR spectra provide useful information about heparin structural features and substitution patterns.⁸ Nitrogen-15 NMR has proved less useful at natural abundance levels, although strategies to enrich the abundance of this nucleus in bacterial or mammalian cell culture have demonstrated the

value of [^1H , ^{15}N] HSQC spectra for GAG characterization.^{3, 9} Previous studies using ^{15}N NMR have been largely limited to characterization of the *N*-acetylglucosamine (GlcNAc) residues prevalent in chondroitin sulfate, dermatan sulfate, HS and hyaluronic acid,^{9, 10} and present in much lower amounts in heparin. To our knowledge, [^1H , ^{15}N] HSQC spectra had not been previously reported for heparin characterization prior to this work, even for the GlcNAc residues.

Like the backbone amide protons of peptides and proteins, the nitrogen-bound protons of GlcNAc exchange slowly with water in aqueous solution due to the partial double-bond character of the amide bond.^{9, 11, 12} As a result, these amide protons give rise to sharp resonances easily detectable in the ^1H NMR spectra of GAGs. It is not surprising that ^{15}N NMR has worked best for semi-synthetic heparin prepared from labeled heparosan which has a fairly regular structure,^{2, 3} and for GAGs such as chondroitin sulfate and hyaluronic acid that are less heterogeneous than heparin and HS and exclusively contain *N*-acetylated galactosamine or glucosamine residues, respectively.^{12,}

13

In addition to the analysis of the amide groups within GAGs, we are interested in the characterization of the sulfamate groups due to their role in the myriad of biological activities mediated by heparin and HS. Prior to our experiments, it was widely believed that in aqueous solution the solvent exchange rate of the sulfamate proton was too fast to permit detection by NMR, therefore an early goal of this project was to determine solution conditions that would minimize the exchange rate and allow measurement of NMR spectra containing resonances of the sulfamate group protons. This was

accomplished by determining solution pH values in which the solvent exchange of the sulfamate protons is slow on the NMR time scale, and allowed for the determination of ^{15}N chemical shifts of the sulfamate moiety at natural abundance levels using the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC experiment.

With the newfound ability to directly detect the nitrogen-bound protons of the glucosamine sulfamate groups,¹⁴ we then take the next step to connect the ^{15}N chemical shifts measured through the one-bond $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ NMR correlation experiments to the other protons in the glucosamine ring using the two-dimensional $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC-TOCSY pulse sequence. In this work, we demonstrate the use of the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC-TOCSY experiment to take advantage of the ample dispersion of the ^{15}N dimension and the efficient magnetization transfer of the TOCSY spin lock to allow observation of correlations for all of the carbon bound protons of the glucosamine ring permitting full resonance assignments for the GlcNS and GlcNAc monosaccharides as well as the three GlcNS residues of the synthetic pentasaccharide drug Arixtra. These HSQC-TOCSY results are compared to our prior work using long-range couplings between ^{15}N and nearby carbon-bound protons with the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ IMPACT-HNMBC experiment which was used to facilitate the assignment of previously unknown ^{15}N chemical shifts of the monosaccharide building blocks of the various GAGs.¹⁵ In the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ IMPACT-HNMBC spectrum, correlations from ^{15}N to H1, H2, and H3 of the glucosamine ring are observed, however, longer-range couplings were not resolved and the very small multiple-bond ^1H - ^{15}N coupling constants (3 Hz) contribute to the poor sensitivity of this approach.

2.2 Methods and Materials

2.2.1. Materials and reagents. D-glucosamine-2-*N*-sulfate, *N*-acetyl-D-glucosamine, D-glucosamine-3-*O*-sulfate and the NMR chemical shift reference standard 2,2-dimethyl-2-silapentane-5-sulfonate-*d*₆ sodium salt (DSS) were purchased from the Sigma Chemical Company (St. Louis, MO). Arixtra (Fondaparinux sodium) was obtained from the University Pharmacy and Department of Pharmacy Administration of Semmelweis University, formulated as prefilled syringes for clinical use. The Arixtra solutions were pooled, desalted on a 1.6 x 70 cm Sephadex G10 superfine column (GE Healthcare, Pittsburgh, PA) using HPLC grade water (Honeywell Burdick & Jackson) as the eluent with a flow rate of 0.15 mL/min. Desalted Arixtra was lyophilized and stored at -20 °C until used.

Samples analyzed using the [¹H, ¹⁵N] IMPACT-HNMBC experiments were prepared in 100% D₂O without adjustment of the solution pH. All other samples were prepared in a 50 mM dibasic sodium phosphate (Fisher Scientific, Pittsburgh, PA) solution containing 90% H₂O (HPLC grade, (Honeywell Burdick & Jackson, Morristown, NJ) and 10% D₂O (Sigma). Solution pH was adjusted using DCl and NaOD (Sigma) also dissolved in 90% H₂O/10% D₂O. The pH meter was calibrated with aqueous buffers at pH 4.00, 7.00 and 10.00 (Fisher) and values are reported without correction for the deuterium isotope effect.

2.2.2. NMR parameters. The ¹H NMR spectra of GlcNS measured as a function of pH were collected using WATERGATE W5 solvent suppression (zgpgw5) into 65536

complex data points using a 7183 Hz spectral window. The WATERGATE delay of 121 μ s was optimized for the detection of the sulfamate ^1H resonances. A Bruker Avance NMR spectrometer operating at 599.79 MHz and equipped with a BBI probe was used for these measurements. The spectra were acquired using a 90° pulse with a length of 8.55 μ s and a 2.0 s relaxation delay. Prior to Fourier transformation, the free induction decays (FIDs) were zero-filled to 65536 points and apodized by multiplication with an exponential function equivalent to a line broadening of 4.0 Hz for the sulfamate region or 0.7 Hz for the spectral region containing the carbon-bound H2 resonances.

The [^1H , ^{15}N] HSQC spectrum of GlcNS was acquired with the Bruker pulse sequence hsqcgpph using States-TPPI.^{34,35} The spectrum was acquired into 4096 complex points in t_2 with 128 scans coadded at each of the 160 t_1 increments. A 2 s relaxation delay was used and an optimized sulfamate $^1J_{(\text{NH})}$ of 80 Hz was determined by acquisition of an [^1H , ^{15}N] HSQC spectrum of GlcNS without ^{15}N decoupling. Spectral windows of 6613 Hz in F2 and 1519 Hz in F1 were employed. Pulse lengths for the ^1H and ^{15}N 90° pulses were 8.63 μ s at -5 dB and 50 μ s at -4 dB, respectively. ^{15}N GARP decoupling during acquisition used a 200 μ s ^{15}N pulse at 10 dB. The spectrum was zero-filled to 4096 x 512 data points and apodized using a $\cos^2$ window function. The ^1H spectrum shown at the top of the contour plot was collected in one scan with the single pulse (zg) experiment using a 90° pulse of 8.63 μ s at -5 dB and a 2 s relaxation delay. Prior to Fourier transformation, the FID was zero-filled to 65536 data points and multiplied by an exponential function equivalent to 0.5 Hz line broadening. ^1H and ^{15}N chemical shifts are referenced relative to DSS which has a ^1H chemical shift of 0.00 ppm.

The [^1H , ^{15}N] HSQC spectrum of Arixtra was measured as described above for GlcNS except that 256 scans were coadded at each of 100 t_1 increments. Pulse lengths for the ^1H and ^{15}N 90° pulses were 9.85 μs at -5 dB and 50 μs at -4 dB, respectively. The data were zero-filled to 8192 x 512 points and linear prediction employed in F1. Apodization using a $\cos^2$ window function preceded Fourier transformation. The WATERGATE W5 (*zgpgw5*) spectrum of Arixtra is plotted along the top of the contour plot. The WATERGATE delay of 300 μs was optimized for detection of the sulfamate ^1H resonances. A total of 32 scans were acquired into 32768 complex data points using a spectral window of 6613 Hz and a 10.0 s relaxation delay. Prior to Fourier transformation, the FID was zero-filled to 65536 data points and multiplied by an exponential function equivalent to 0.5 Hz line broadening.

The [^1H , ^{15}N] HSQC-TOCSY spectrum measured for the 100 mM solution of GlcNS and GlcNAc was acquired using a modified version of the Bruker *hsqcddtgp* pulse sequence using echo-anti-echo phase cycling and modified to incorporate a gradient pulse during the INEPT transfer.^{16, 17} The spectrum was acquired into 4096 complex points in t_2 with 256 scans coadded for each of the 90 t_1 increments. A 1.5 s relaxation delay was used with a $^1J_{(\text{NH})}$ of 80 Hz. The low power 90° pulse used for the TOCSY DIPSI¹⁸ spin lock was 40 μs at 7.8 dB with a mixing time of 150 ms. Spectral windows of 6613 Hz in F2 and 2734 Hz in F1 were employed. The 90° pulse lengths were 9.35 μs at -5.0 dB and 50 μs at -4 dB for ^1H and ^{15}N , respectively. Decoupling was performed during acquisition using GARP at 10.00 dB with a 200 μs pulse. A trim pulse of 0.10 μs was used. Gradient pulses were applied using the shape SMSQ10.100 at 80%, 8.1%, and -

45% of maximum power (5.35 G/mm) for GPZ1 GPZ2, GPZ3 respectively, and a 1 ms homospoil pulse was applied. The spectrum was zero-filled to 8192 x 512 data points and apodized using a $\cos^2$ window function in both dimensions. The ^1H spectrum plotted at top of the contour plot was collected in 16 scans into 65536 data points using WATERGATE W5 solvent suppression (*zgpgw5*) with a WATERGATE delay of 121 μs and a 2.0 s relaxation delay. This spectrum was zero-filled to 65536 points.

The [^1H , ^{15}N] HSQC-TOCSY spectrum of a 40 mM solution of Arixtra in 90% H_2O /10% D_2O , 25 mM phosphate buffer at pH 8.10 was acquired as described for the GlcNS/GlcNAc solution except that 368 scans were coadded for each of the 96 t_1 increments and a 1.5 s relaxation delay was used. The *hsqcdietgpsi* pulse sequence was modified to incorporate WATERGATE W5 solvent suppression just prior to acquisition.¹⁹ For the more concentrated solution of GlcNS and GlcNAc, gradient selection of signal quantum coherence pathways provided sufficient water suppression, however for the Arixtra solution, interference from the F1 noise band produced by the residual water resonance interfered with the observation of some cross peaks. In addition, by using solvent suppression a higher level of receiver gain could be used which aided in the detection of the weaker cross peaks of the H6 resonances. The value of $^1J_{(\text{NH})}$ (82 Hz) was determined from an [^1H , ^{15}N] HSQC spectrum measured without ^{15}N decoupling. The low power pulse used for the TOCSY spin lock was 40 μs at 8.0 dB and 90° pulse lengths of 10.00 μs at -4.0 dB and 39 μs at -4.4 dB were used for ^1H and ^{15}N , respectively. Spectral windows of 6613 Hz in F2 and 1000 Hz in F1 were employed. The spectrum was zero-filled to 8192 x 1024 data points and apodized using a $\cos^2$ window

function in both dimensions. The spectrum was forward linear predicted to 128 points. The ^1H spectrum plotted at the top of the contour plot was collected in 32 scans using WATERGATE W5 solvent suppression (*zgpgw5*) with 65536 data points, a 90° pulse of 10.00 μs at -4 dB, a 2 s relaxation delay and a WATERGATE delay of 200 μs . The spectrum was zero-filled to 65536 points.

The [^1H , ^{15}N] IMPACT-HNMBC spectrum measured for a 100 mM solution of GlcNS/GlcNAc in D_2O was acquired using the pulse sequence described previously incorporating echo-anti-echo phase cycling.¹⁵ The spectrum was acquired into 4096 complex points in t_2 with 256 scans coadded for each of 90 t_1 increments. A 2.0 s relaxation delay was used with a long range coupling constant of 3 Hz. Spectral windows of 6613 Hz in F2 and 2734 Hz in F1 were employed. The 90° pulse lengths of 9.18 μs at -5.0 dB and 50 μs at -4 dB were used for ^1H and ^{15}N , respectively. The spectrum was zero-filled to 4096 x 1024 data points and apodized using a $\cos^2$ window function in F2 and a sin window function in F1. The ^1H spectrum plotted at the top of the contour plot was collected in 8 scans using the single pulse (*zg*) experiment with a 90° pulse of 9.18 μs at -5 dB and a 2 s relaxation delay.

The [^1H , ^{15}N] IMPACT-HNMBC of the 40 mM Arixtra sample in D_2O was acquired as described for the GlcNS/GlcNAc solution except that the spectrum was acquired into 2048 complex points in t_2 with 960 scans coadded for each of the 64 t_1 increments. Spectral windows of 3004 Hz in F2 and 911 Hz in F1 were employed. The 90° pulse lengths were 9.31 μs at -5.0 dB and 50 μs at -4 dB for ^1H and ^{15}N , respectively. The spectrum was zero-filled to 4096 x 256 data points. The ^1H spectrum plotted at the

top of the contour plot was collected in 16 scans using the single pulse (*zg*) experiment with a 90° pulse of 9.31 μ s at -5 dB and a 4 s relaxation delay. Prior to Fourier transformation the FID was zero-filled to 131072 points.

The [^1H , ^{15}N] IMPACT-HNMBC spectrum of Arixtra measured at 800 MHz was performed by Dr. Szabolcs Beni from the University Pharmacy and Department of Pharmacy Administration of Semmelweis University using a cold probe. The sample prepared by pooling 5 Arixtra ampules, lyophilizing and reconstituting in 600 μ L D_2O containing 2 mM DSS. NMR analysis was performed in a salt tolerant S-tube using the ^{15}N -gHMBC pulse program. The spectrum was acquired into 4096 complex points in t_2 with 128 scans coadded for each of the 96 t_1 increments. The 90° ^1H pulse used was 7.1 μ s at 57 dB and the ^{15}N pulse was 35.1 μ s at 57 dB. A multiple bond correlation value of 4 Hz was used. The spectrum was zero-filled to 8K points and no linear prediction was used in F1

The [^1H , ^{15}N] MPACT-HNMBC of GlcN(3S) was acquired similarly to the parameters used for the GlcNS/GlcNAc mixture except that the spectrum was acquired into 2048 complex points in t_2 with 128 scans coadded for each of the 256 t_1 increments. Spectral windows of 2693 Hz in F2 and 1823 Hz in F1 were employed. The 90° pulse lengths were 8.12 μ s at -5.0 dB and 50 μ s at -4 dB for ^1H and ^{15}N , respectively. The spectrum was zero-filled to 2048 x 1024 data points and then apodized using a $\cos^2$ window function in F2 and sin window function in F1. The ^1H spectrum plotted at the top of the contour plot was collected in 8 scans using the single pulse (*zg*) experiment with a 90° pulse of 8.12 μ s at -5 dB and a 1.5 s relaxation delay.

2.3. Results and Discussion

2.3.1 Detection of sulfamate resonances by ^1H NMR. We addressed the challenge of detecting the sulfamate protons in aqueous solution by building on the large body of work involving NMR measurements of the amide protons of peptides and proteins and the nitrogen-bound protons of nucleotide bases, the building blocks of DNA and RNA.²⁰⁻²² The exchange rates of these classes of nitrogen-bound protons are highly pH dependent and pH optima of 3–4 for detection of peptide backbone amide protons and pyrimidine nucleotides, and 6–8 for purine nucleotides have been identified.²² Shown in Figure 2.1 are the pH dependent ^1H NMR spectra of 5 mM GlcNS measured in 90% $\text{H}_2\text{O}/10\% \text{D}_2\text{O}$ at 25 °C using WATERGATE W5 solvent suppression.¹⁹ Due to solvent exchange of the sulfamate protons, water suppression using selective saturation or even WET significantly reduces the intensity of these resonances, even at the pH minimum for exchange. WATERGATE W5 solvent suppression has a minimal impact on the sulfamate proton resonance intensities.¹⁹ GlcNS is present in solution in both the α and β anomeric forms which appear, from Figure 2.1, to have minima in their exchange rates at slightly different pH values. The initial assignment of the α (5.34 ppm) and β (5.79 ppm) sulfamate group ^1H resonances was based on their relative intensities, which are consistent with those of the carbon-bound anomeric H1 resonances.

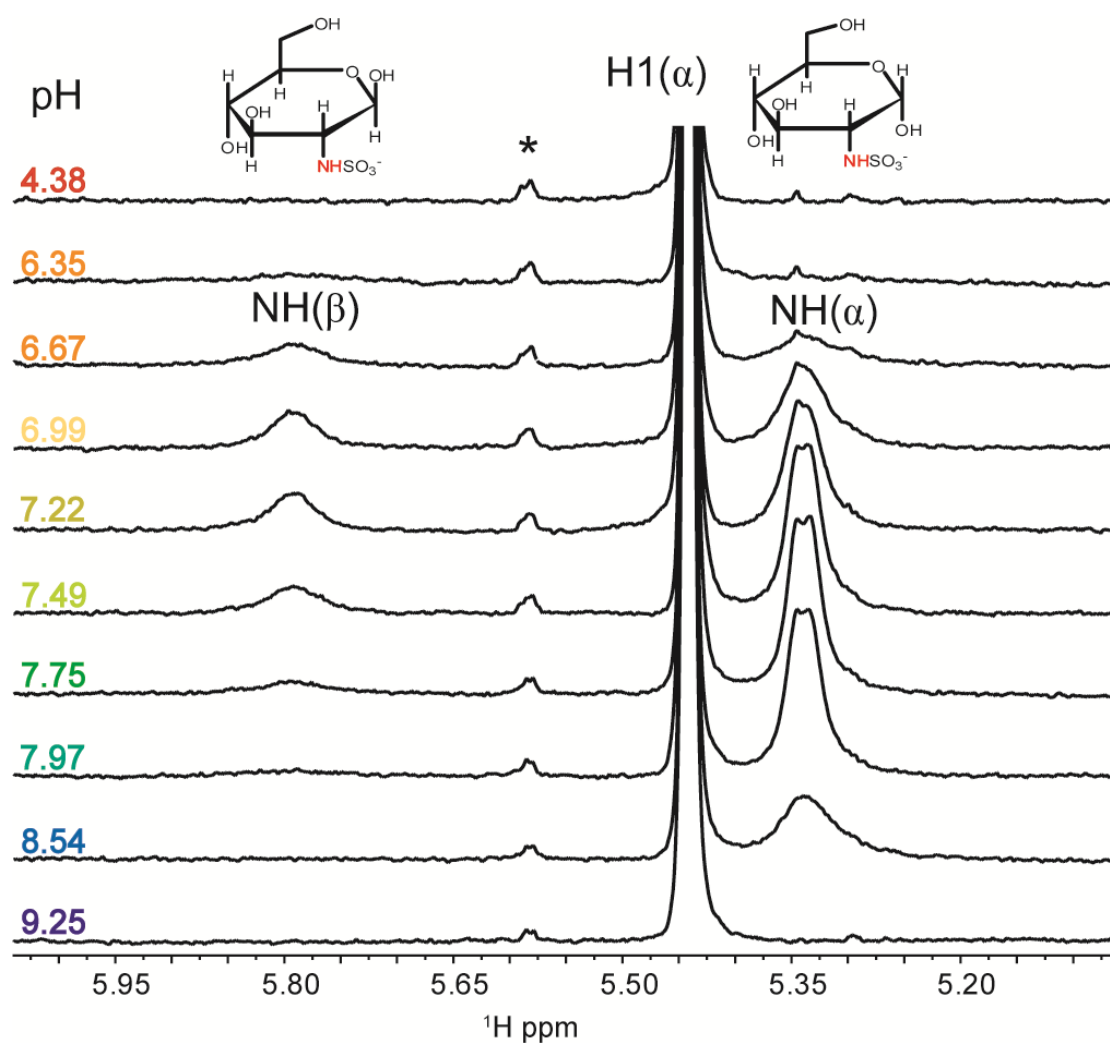


Figure 2.1. Selected regions of the ^1H NMR spectra of *N*-sulfo-*D*-glucosamine (GlcNS) measured as a function of pH in 90% H_2O /10% D_2O at 25 $^\circ\text{C}$ using WATERGATE W5 solvent suppression. The sulfamate proton resonances of the GlcNS α (5.34 ppm) and β (5.79 ppm) anomers are indicated. The asterisk marks the carbon-bound proton resonance of an impurity.

At pH 7.49, the rate of exchange of the sulfamate proton with H₂O is sufficiently slow that the NH resonance of the α anomer reflects the coupling to the adjacent GlcNS H2. The rate of NH chemical exchange also has a pronounced effect on the peak width of the adjacent H2 resonances as can be discerned by examination of Figure 2.2. Below pH 6 and above pH 9 where the rate of exchange of the sulfamate protons with water is rapid, the H2 resonances are sharp. At pH values where the exchange rate begins to slow and a broad NH resonance is detected, broadening can also be seen for the coupled H2 resonances. For the α anomer (Figure 2.2A), the H2 resonance in the spectrum measured at pH 7.5 where the rate of exchange is slowest, is consistent with that of a complex multiplet including the NH coupling of 6 Hz. At this temperature the β anomer sulfamate resonance never sharpens sufficiently to resolve its coupling to H2 (Figure 2.1).

By measuring ¹H NMR spectra of GlcNS as a function of pH we determined that the sulfamate ¹H resonances are detectable in the slightly basic pH regime. Examination of the resonances of the H2 proton, which are scalar coupled to the sulfamate NH, we further substantiated the assignment of the resonances at 5.34 ppm and 5.79 ppm to the NHSO₃⁻ protons of the GlcNS α and β anomers, respectively. This result was unexpected based on published proton exchange rates of protein and peptide amide protons, which have solvent exchange minima in acidic pH regimes.

In aqueous solution, the process of proton exchange contains elements of both the acid and base catalyzed reactions and the pH dependence can be expressed using Eq. 1 as described by Englander et al.²³ and Woodward et al.²⁴ It is important to note that while

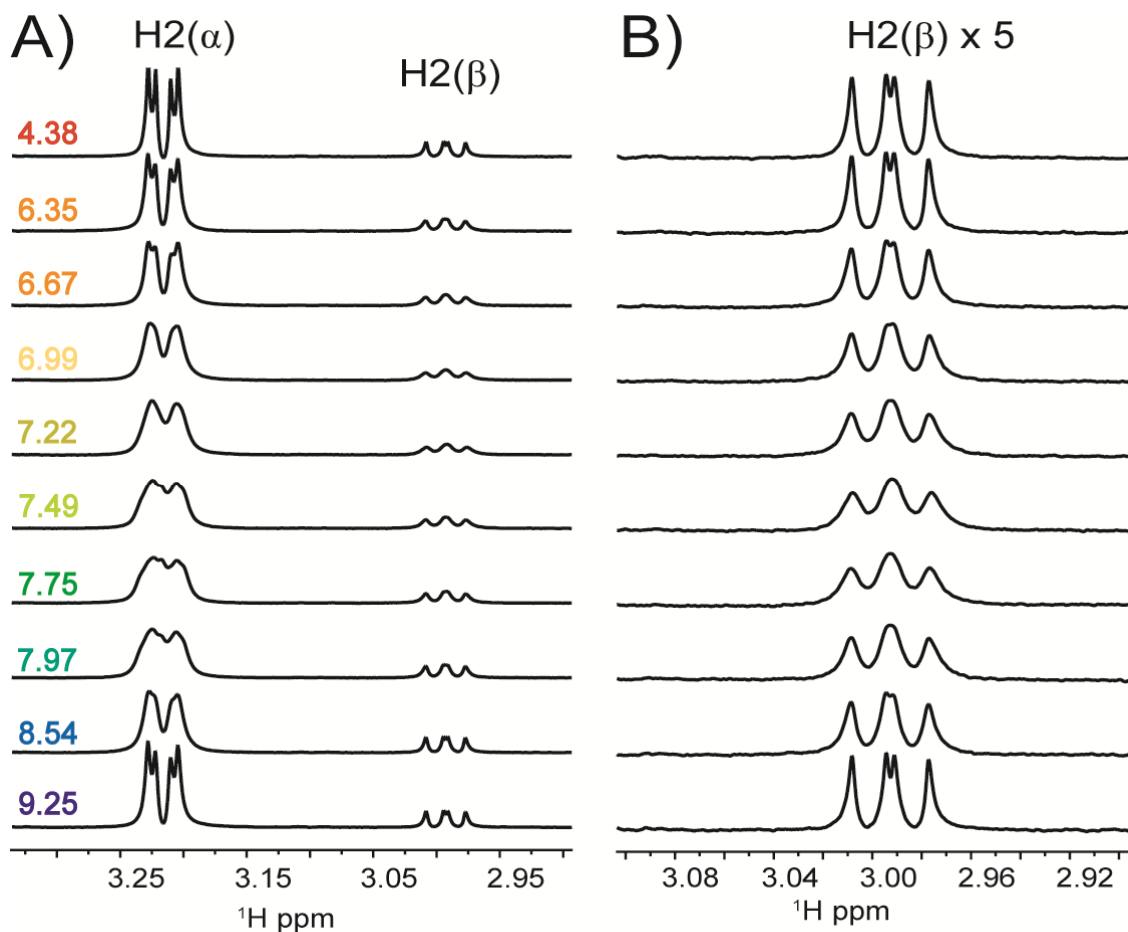


Figure 2.2. Selected regions of the ^1H NMR spectra of *N*-sulfo-*D*-glucosamine (GlcNS) measured as a function of pH in 90% H_2O /10% D_2O at 25 °C using WATERGATE W5 solvent suppression. A) The spectral region containing the GlcNS H2 resonances showing the effect of the sulfamate proton chemical exchange. B) A 5-fold scale enhancement of the H2 resonance of the β anomer.

either the acid or base catalyzed reaction may dominate in their contribution to the observed proton exchange rate both take place to some degree in aqueous solution

$$k_{\text{obs}} = k_{\text{H}}[\text{H}_3\text{O}^+] + k_{\text{OH}}[\text{OH}^-] \quad \text{Eq. 1}$$

where k_{obs} is the observed proton exchange rate, k_{H} is the acid-catalyzed exchange rate and k_{OH} is the base-catalyzed exchange rate. Acid-catalyzed exchange occurs through a mechanism initiated through *N*-protonation by H_3O^+ to produce a quaternary cationic intermediate, followed by deprotonation by H_2O to regenerate H_3O^+ , as depicted in Figure 2.3A. The mechanism of base-catalyzed exchange involves *N*-deprotonation by OH^- to form the imidate ion, which is then reprotonated by H_2O to regenerate OH^- (Figure 2.3B).²⁵

In the case of amides, the observed exchange rate is minimized in the pH range 3 - 4 suggesting that acid-catalyzed exchange makes a small contribution to the overall exchange rate under these conditions. Molday and Kallen reported that the main mode of proton exchange for amides is through the base-catalyzed-imidic acid mechanism, whereby protonating the amide carbonyl lowers the pK_a of the *O*-protonated imidic acid from an estimated 18 for *N*-methylacetamide to around 7.5 for *O,N*-dimethylacetimidate, allowing for deprotonation and base-catalyzed exchange at pH values achievable in aqueous solution (Figure 2.3C).²⁶

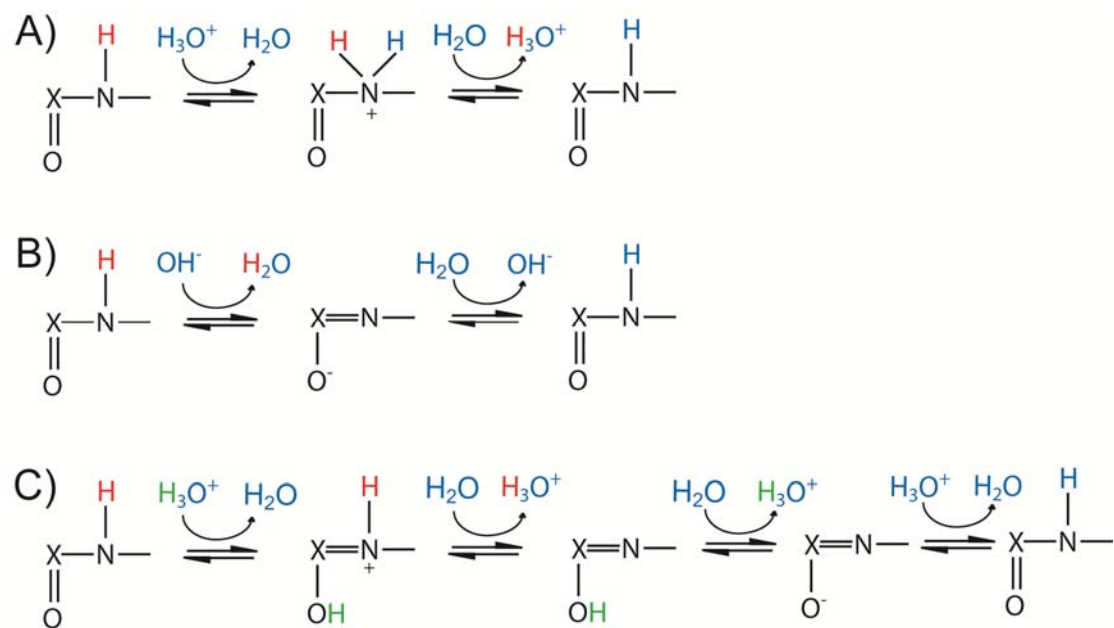


Figure 2.3. Proton exchange mechanisms. A) acid-catalyzed proton exchange, B) base-catalyzed proton exchange, C) imidic acid proton exchange mechanism. In (A) and (B) X can be C or S. In (C) X = C.

While much less is known about the proton exchange of sulfamate groups, it is possible that the absence of the carbonyl group forces the exchange to occur through alternative pathways. It is also likely that the anionic charge of the proximal *N*-sulfo group influences the hydroxide mediated base-catalyzed exchange through electrostatic repulsion of the hydroxide ions. Additionally, it is observed that the hydroxyl groups of GlcNS may play a significant role in the sulfamate proton exchange process as we performed a pH titration on the artificial sweetener sodium cyclamate (sodium *N*-cyclohexylsulfamate) and no NH peaks were observed over a pH range from 4 to 11 (data not shown).

2.3.2 Detection of sulfamate resonances by [^1H , ^{15}N] HSQC NMR. With solution conditions in place to detect the sulfamate ^1H NMR resonances, indirect detection of ^{15}N chemical shifts via the [^1H , ^{15}N] HSQC NMR experiment becomes feasible.^{14, 15} Figure 2.4 shows the [^1H , ^{15}N] HSQC spectrum measured at 5 °C for 5 mg GlcNS dissolved in 40 μL of 90% H_2O /10% D_2O , 50 mM phosphate buffer, pH 7.5 using a 1.7 mm OD coaxial insert NMR tube. An average $^1J_{(\text{NH})}$ of 80 Hz was used and 160 t_1 increments were acquired. Reducing the temperature to 5 °C reduces the rate of $\text{NH}-\text{H}_2\text{O}$ exchange, sharpening the sulfamate proton resonances of both GlcNS anomers compared with Figure 2.1.

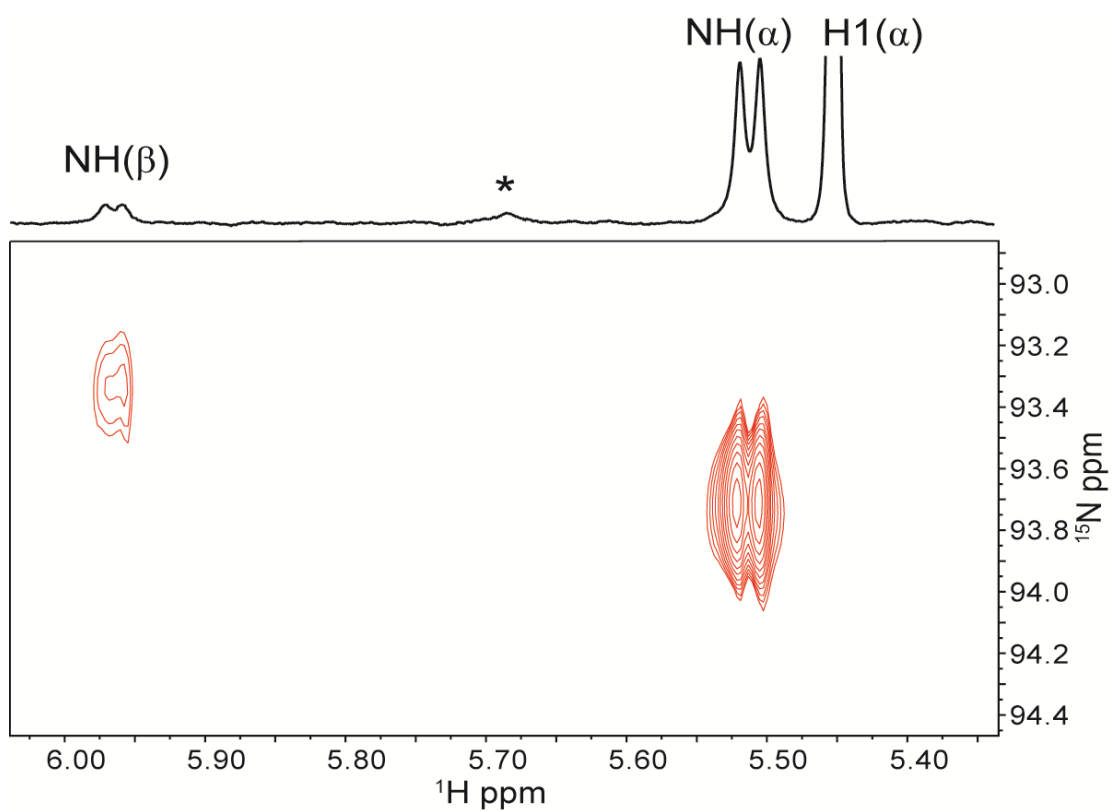


Figure 2.4. [^1H , ^{15}N] HSQC spectrum of GlcNS at pH 7.5 and 5 °C. The asterisk marks the carbon-bound proton resonance of an impurity.

The successful acquisition of the [^1H , ^{15}N] HSQC spectrum of GlcNS under conditions where the sulfamate ^1H resonances are detectable proves that the resonances in question are indeed due to nitrogen bound protons. The different chemical shifts of the ^{15}N nuclei ($\alpha = 93.71$ ppm, $\beta = 93.32$ ppm) shown in Figure 2.4 are consistent with the values we reported previously through long-range ^{15}N – ^1H couplings measured with the [^1H , ^{15}N] IMPACT-HMBC experiment using carbon-bound protons, and confirms the ^1H resonance assignments of the two GlcNS anomers.²⁷

2.3.3. ^1H and ^{15}N NMR measurements of the sulfamate groups of the pentasaccharide Arixtra. Although detection of the GlcNS sulfamate ^1H and ^{15}N resonances is interesting, to be useful for GAG characterization the ^1H and/or ^{15}N NMR chemical shifts must be sensitive to subtle structural differences. We selected the synthetic heparin mimetic Arixtra to probe the potential value of [^1H , ^{15}N] HSQC NMR spectra (Figure 2.5) for heparin and HS structural characterization. The structure of Arixtra is closely related to the native heparin pentasaccharide responsible for binding to antithrombin III. The Arixtra pentasaccharide, shown in Figure 2.5A, contains three distinct sulfamate groups all in different local chemical environments. From a series of ^1H NMR survey spectra measured using WATERGATE W5 solvent suppression as a function of pH, we determined a pH optimum of 8.2 for detection of the NHSO_3^- protons of Arixtra. The pH and temperature dependence of sulfamate proton exchange in Arixtra is discussed in detail in Chapter 3.

The anomeric forms of GlcNS provide an example of constitutional isomers, for which the sulfamate group's ^1H and ^{15}N chemical shifts of each anomeric form are

distinct. This observation is a promising lead for the application of the [^1H , ^{15}N] HSQC experiment as a new tool for heparin characterization. It is, however, important that the reflection of structure on the ^1H and ^{15}N chemical shifts be carried beyond the anomeric forms of monosaccharides to more complex samples such as oligosaccharides. The [^1H , ^{15}N] HSQC spectrum of Arixtra (Figure 2.5B) shows that each of the sulfamate ^1H and ^{15}N resonances of Arixtra has unique chemical shifts. Starting with the first residue of the non-reducing end GlcNS(6S), labeled as (I), we observe ^1H and ^{15}N chemical shifts of 5.99 ppm and 92.66 ppm, respectively. The central GlcNS(3S)(6S) residue, labeled as (III), has shifts of 5.58 ppm and 91.90 ppm respectively and the terminal residue containing the methyl ester, labeled as (IV) has shifts of 5.55 ppm 93.59 ppm. The high concentration of Arixtra in this sample and the lower analysis temperature allows for sharper peaks in the [^1H , ^{15}N] HSQC spectrum with clear *J*-coupling provides more confidence in the assignment of the ^{15}N chemical shifts to 2 decimal places. The results in Figure 2.5 indicate that both the ^1H and ^{15}N chemical shifts of the sulfamate groups can report on the local environment of an individual GlcNS residue, consistent with similar observations for the amide resonances of GlcNAc residues in other GAGs.^{2,}

14, 15

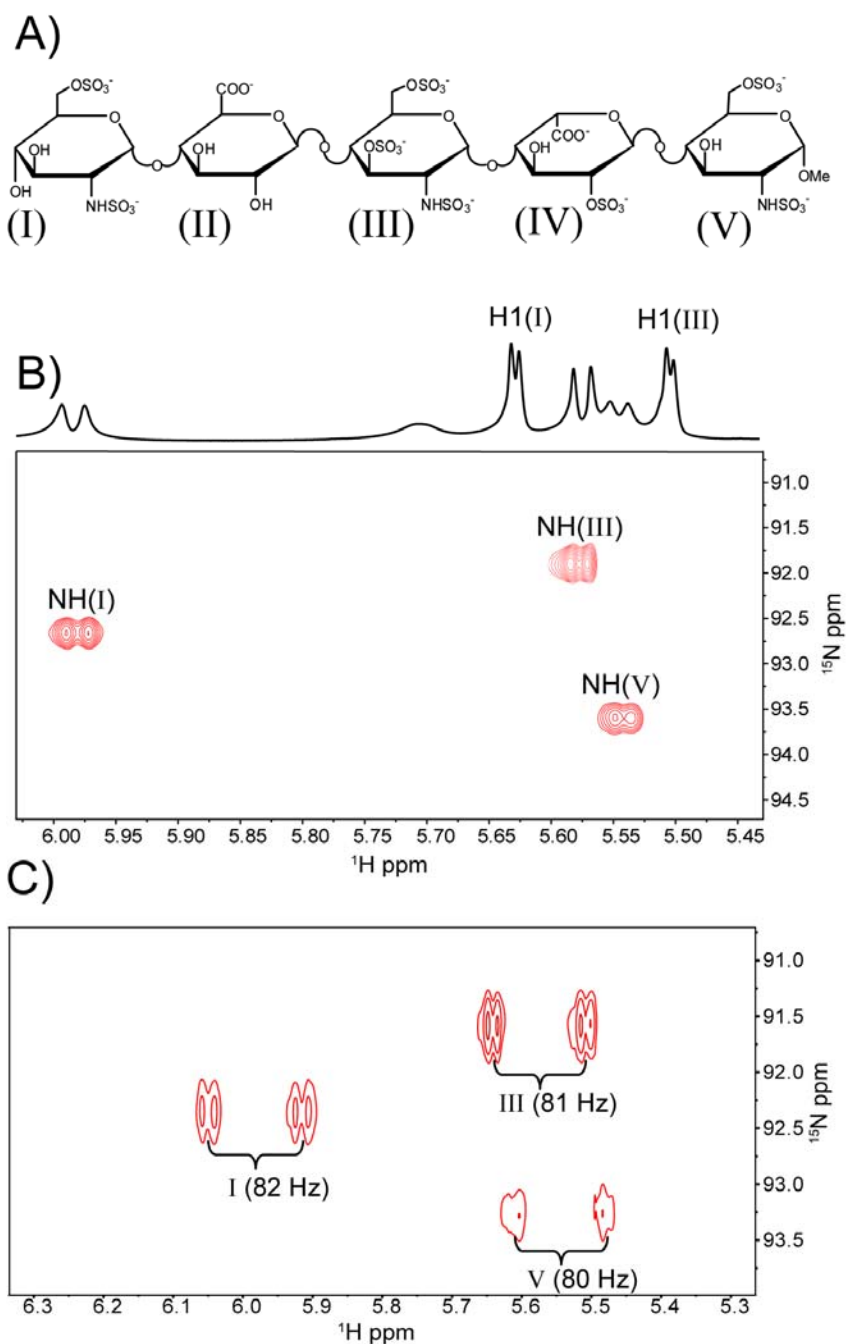


Figure 2.5. A) Structure of Arixtra. B) $[^1\text{H}, ^{15}\text{N}]$ HSQC spectrum of Arixtra in 90% $\text{H}_2\text{O}/10\%$ D_2O in 20 mM phosphate buffer at pH 8.2 and 5 °C. The ^1H WATERGATE W5 spectrum is plotted at the top of the contour map showing the carbon-bound anomeric proton resonances of GlcNS residues I and III in addition to the sulfamate proton resonances. C) $[^1\text{H}, ^{15}\text{N}]$ HSQC spectrum of Arixtra measured without ^{15}N decoupling. The measured $^1J_{(\text{HN})}$ values are displayed for each sulfamate group.

Measuring the [^1H , ^{15}N] HSQC spectrum of Arixtra without the use of ^{15}N decoupling results in a spectrum in which the peaks are split in the F2 dimension (Figure 2.5C). This splitting reflects the $^1J_{(\text{HN})}$ heteronuclear coupling and allows for the accurate measurement of these values for each sulfamate cross peak. Determining the experimental $^1J_{(\text{HN})}$ values for each sulfamate group allows for optimal sensitivity of the [^1H , ^{15}N] HSQC experiment as this value is used to calculate the time dependant delay for magnetization transfer, defined as $1/(4*^1J_{(\text{HN})})$. Additionally, the measurement of the couplings demonstrates the consistency of the sulfamate $^1J_{(\text{HN})}$ and the appropriateness of the values used.

2.3.4 2D [^1H , ^{15}N] HSQC-TOCSY as a potential tool for heparin characterization.

Complete resonance assignments for the GlcN residues of the biologically important and structurally complex GAGs heparin and HS requires information beyond the sulfamate and amide ^1H and ^{15}N chemical shifts provided by the HSQC spectra. In this section we explore the potential of the [^1H , ^{15}N] HSQC-TOCSY experiment²⁸ to provide complete assignments for variously substituted glucosamine residues. To achieve this goal, we used a solution containing 100 mM of the monosaccharides GlcNS and GlcNAc to optimize the experimental conditions for detection of TOCSY correlations from the sulfamate group NH to all of the carbon-bound protons within a monosaccharide ring (Figure 2.6).

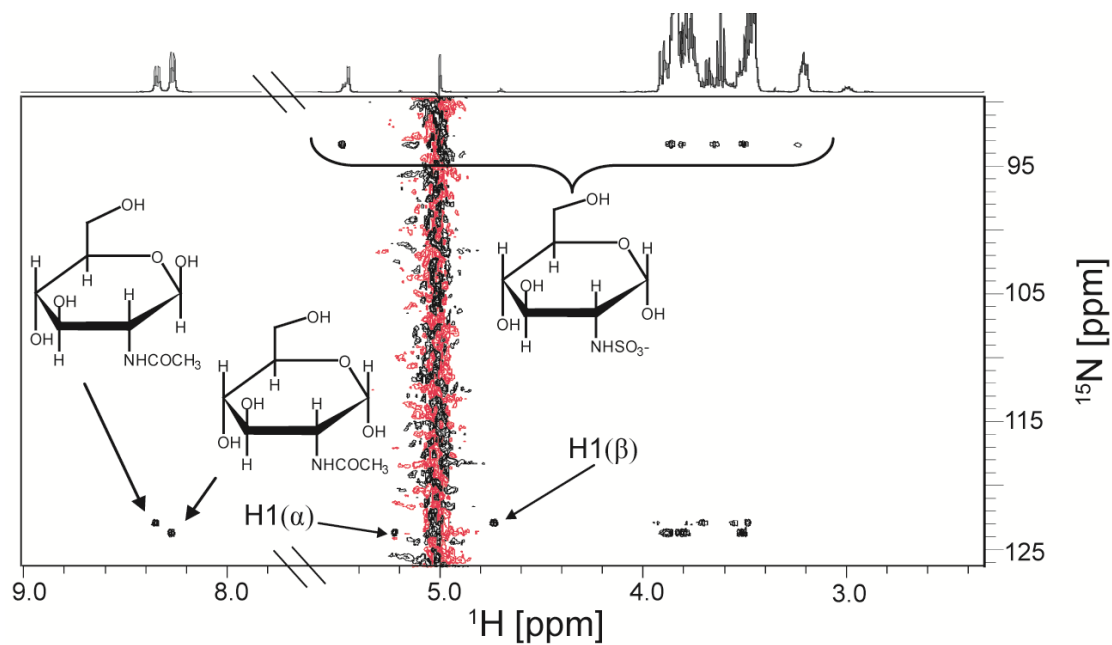


Figure 2.6. [^1H , ^{15}N] HSQC-TOCSY spectrum of 100 mM GlcNS and GlcNAc in 90% H_2O /10% D_2O at 5 $^\circ\text{C}$ and pH 7.48.

Solution conditions are critical for [^1H , ^{15}N] HSQC-TOCSY experiments, in which detection of nitrogen-bound protons is the crux of the experiment. The optimum solution pH is limited by the narrow range over which the sulfamate protons are in slow exchange with water on the NMR time scale. We selected pH 7.48 for the measurement of the HSQC-TOCSY spectrum of 100 mM GlcNS and GlcNAc in 90% H_2O /10% D_2O because this value corresponds to the exchange minimum for the less intense GlcNS β anomer. Even at pH 7.48, the GlcNS sulfamate protons undergo more rapid solvent exchange than do the amide protons of GlcNAc which appear as sharp doublets in Figure 2.6. While the amide protons of GlcNAc exchange more slowly at lower pH, their relative exchange rates are still slower at neutral pH (far from their optimum range) than the exchange rates of the GlcNS sulfamate protons at their optimal pH.

For GlcNAc, the two TOCSY rows of the α - and β -anomers are nicely separated with distinct ^{15}N chemical shifts. Because the acetyl methyl protons are not coupled to the protons of the sugar ring, no TOCSY cross peaks are observed for this resonance. For purposes of resonance assignment, the anomeric protons of H1 (α) and H1 (β) serve as good entry points allowing the unambiguous assignment of the sharp amide doublets. The remaining carbon-bound protons of the sugar ring can be assigned using correlation spectroscopy data or based on literature chemical shift values.²⁷ The HSQC-TOCSY spectrum in Figure 2.6 contains only a single TOCSY row for the GlcNS α -anomer; the peaks of the β -anomer were not detected above the noise floor probably because of its lower abundance and much broader sulfamate proton resonance even at the pH optimum for this residue. The H1 resonance of the GlcNS α -anomer (5.450 ppm) is partially

overlapped with the broader sulfamate NH resonance. The H2 resonance of GlcNS can be easily assigned, as this is the most upfield resonance in both GlcNS anomers.

Assignments of the remaining protons can be made using either the COSY connectivities or the literature chemical shift values.²⁹

To test the effectiveness of the [¹H, ¹⁵N] HSQC-TOCSY experiment for resonance assignments in a more complex molecule, we returned to the Arixtra pentasaccharide (Figure 2.5A). Having GlcNS residues in 3 unique environments with different neighboring saccharides, Arixtra is an excellent example of a biologically relevant heparin-derived oligosaccharide. The [¹H, ¹⁵N] HSQC-TOCSY spectrum of a 40 mM Arixtra solution in 90% H₂O/10% D₂O, 25 mM phosphate buffer at pH 8.10 is shown in Figure 2.7. The best quality spectra were achieved with a modification of the standard Bruker *hsqcetgpsi* pulse sequence to introduce WATERGATE W5 solvent suppression just prior to acquisition along with a gradient pulse in the center of the INEPT transfer to destroy unwanted transverse magnetization.^{16, 17} Solvent suppression was further enhanced by selecting the gradient pulse shape SMSQ10.100 in the HSQC portion of the experiment. This gradient shape results in a sharper water signal which improves its attenuation by the homospoil gradient pulse. This pulse program can be found in Appendix 1.

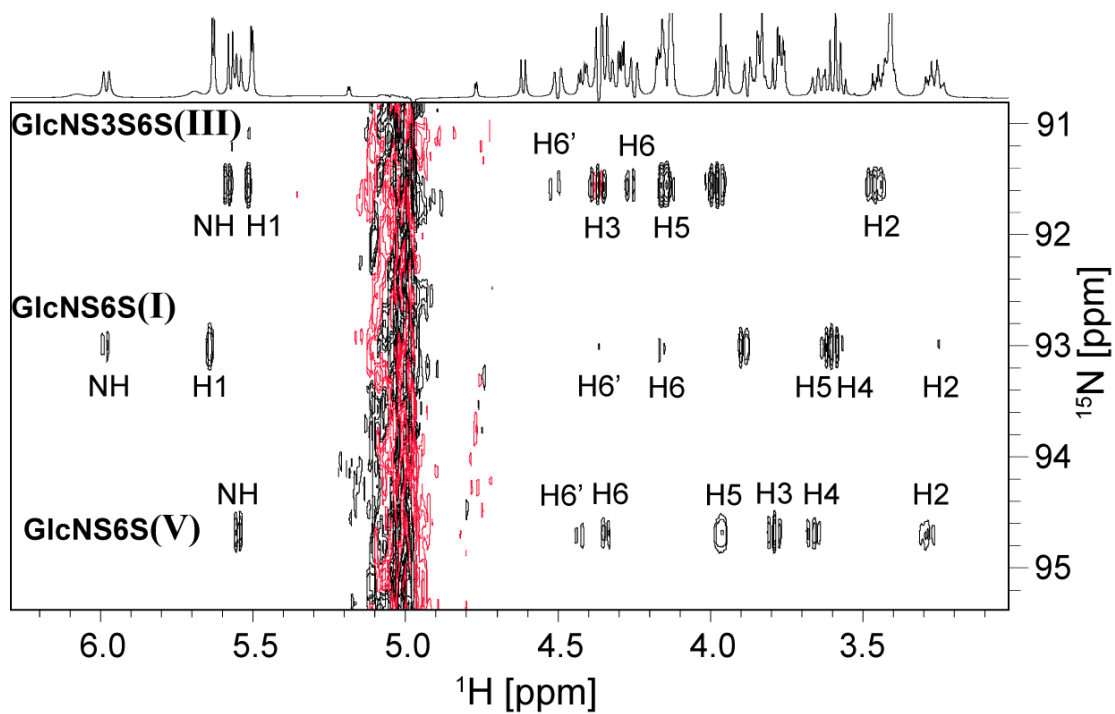


Figure 2.7 [¹H, ¹⁵N] HSQC-TOCSY spectrum measured for a 40 mM Arixtra solution in 90% H₂O/10% D₂O containing 25 mM phosphate at pH 8.10 and 5 °C.

The HSQC-TOCSY spectrum of Arixtra (Figure 2.7) shows the three spin systems of the GlcNS residues nicely resolved in the ^{15}N dimension. The ability to determine the chemical shifts, summarized in Table 2.1, of the complete glucosamine spin system using the HSQC-TOCSY experiment is especially important considering the biochemical importance of 6-*O*-sulfonation and the spectral overlap that can hinder assignments in homonuclear correlation experiments. The cross peak intensities in the TOCSY rows of the *N*-sulfoglucosamine residues depend on the line widths of the sulfamate NH resonances. The strongest cross peaks were detected for the GlcNS(III) residue, for which the NH resonance line width is comparable to those of the Arixtra carbon bound protons, as observed in Figure 2.5B. The GlcNS(I) residue showed the broadest NH resonance and consequently the cross peaks in this TOCSY row are the weakest. However, correlations between ^{15}N and all ring proton resonances were detected even for this residue. The anomeric resonance of the GlcNS(V) residue is attenuated by the solvent suppression scheme and lies in the t_1 noise of the solvent. The detailed assignments of compounds measured with the [^1H , ^{15}N] HSQC-TOCSY experiment is presented in Table 2.1.

Table 2.1 Summary of the ^1H and ^{15}N NMR chemical shifts (in ppm) measured using the $[\text{}^1\text{H}\text{-}^{15}\text{N}]$ HSQC-TOCSY pulse sequence for the compounds studied. Chemical shifts measured using one-dimensional ^1H NMR spectra are reported to three decimal places. Chemical shifts determined from 2D spectra are reported to two decimal places due to the poorer digital resolution of these measurements. Asterisks denote peaks where resonance overlap limits peak resolution.

HSQC-TOCSY	^{15}N	NH	H1	H2	H3	H4	H5	H6	H6'
GlcNAc(α)	124.12	8.266	5.194	3.888	3.763	3.483	3.8*	3.802	3.8*
GlcNAc(β)	123.33	8.342	4.704	4.746	3.533	3.697	3.465	3.747	3.913
GlcNS(α)	93.36	5.469	5.450	3.24	3.65	3.56	3.86	3.79	3.81
Arixtra GlcNS(I)	93.00	5.979	5.629	3.26	3.59	3.43	3.877	4.14	4.36
ArixtraGlcNS(III)	91.53	5.507	5.502	3.44	4.35	3.96	4.14	4.248	4.498
Arixtra GlcNS(V)	94.46	5.545		3.27	3.78	3.65	3.96	4.32	4.40

2.3.5 Comparison of results obtained with the [^1H , ^{15}N] IMPACT-HNMBC and [^1H , ^{15}N] HSQC-TOCSY experiments. The [^1H , ^{15}N] IMPACT-HNMBC experiment reported by Limtiaco et al.¹⁵ and used in this chapter is a modification of the constant-time IMPACT-HMBC pulse sequence published by Furrer.³⁰ To tailor the experiment for our use we removed the *J*-filter used to suppress $^1J_{(\text{NH})}$ correlations and collapsed three existing gradient pulses into a single pulse labeled G3 in Figure 2.8. Additionally, the 180° shaped pulse used by Furrer just prior to acquisition was changed to a standard 180° hard pulse to make the experiment non-selective and allow simultaneous detection of correlations for glucosamine residues with *N*-acetyl and *N*-sulfo substitutions as well as unmodified amine groups. The constant-time aspect of the experiment eliminates ^1H - ^1H homonuclear *J*-evolution resulting from increases in the incremented delay over the course of the experiment.

Although the [^1H , ^{15}N] HSQC-TOCSY spectrum provides a wealth of information and increased sensitivity compared to the [^1H , ^{15}N] IMPACT-HNMBC, it remains limited by the proton exchange rate of the NH groups which are dependent on temperature and pH. When experimental conditions cannot sufficiently slow solvent exchange for [^1H , ^{15}N] HSQC-TOCSY detection, the [^1H , ^{15}N] IMPACT-HNMBC experiment can be used to detect correlations to ^{15}N through long-range couplings to the non-exchangeable

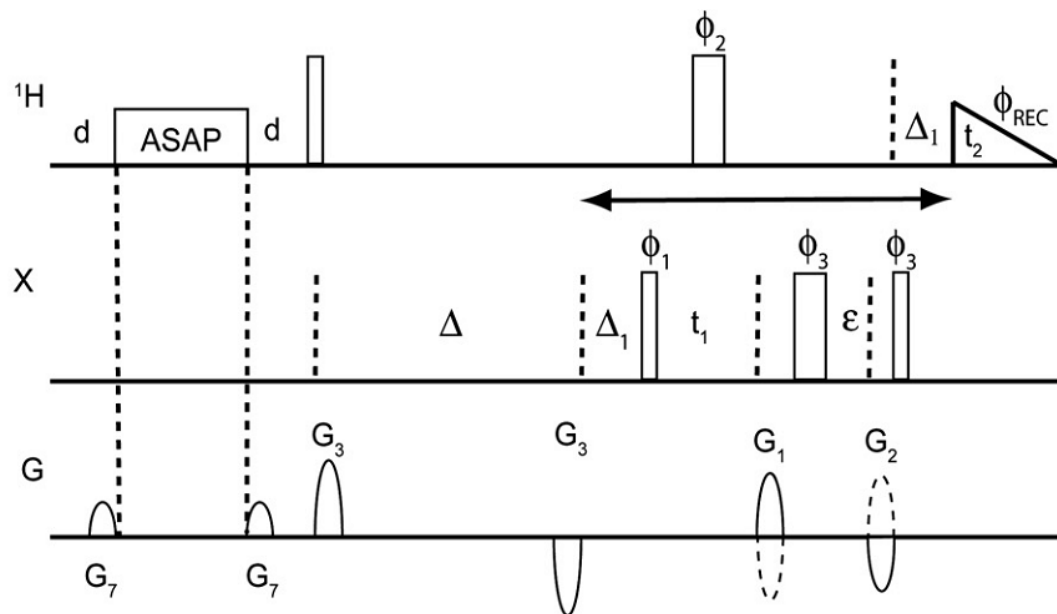


Figure 2.8. Pulse sequence for the acquisition of $[^1\text{H}, ^{15}\text{N}]$ IMPACT-HMBC spectra presented in this research.¹⁵

carbon-bound protons. This section focuses on comparing [^1H , ^{15}N] IMPACT-HNMBC spectra for GlcNAc, GlcNS and Arixtra to those measured by [^1H , ^{15}N] HSQC-TOCSY (Section 2.3.4) and then expanding the application of this approach to a situation where rapid solvent exchange prohibits the measurement of [^1H , ^{15}N] HSQC-TOCSY spectra.

Figure 2.9 displays a single spectrum divided into two regions. The upper strip presents the GlcNS correlations and the bottom strip shows the GlcNAc spectral region. This spectrum was acquired to show the ability of the [^1H , ^{15}N] IMPACT-HNMBC pulse sequence to simultaneously measure correlations for both *N*-sulfated and *N*-acetylated GlcN, in a similar fashion to the [^1H , ^{15}N] HSQC-TOCSY.

In the GlcNAc spectral region of the IMPACT-HNMBC spectrum in Figure 2.9, ^1H - ^{15}N cross peaks to the carbon-bound proton resonances of the α anomer were detected for H2 (3.75 ppm) and H3 (3.87 ppm), while cross peaks for the β anomer were detected for H1 (4.707 ppm), H2 (3.53 ppm), and H3 (3.67 ppm). A single intense peak was observed for the acetyl methyl protons of both anomers at 2.04 ppm. The GlcNAc α and β anomers have different ^{15}N chemical shifts and are well-resolved in this spectrum. For GlcNS, the α anomer predominates (~85%), thus strong correlations are observed only between ^{15}N and the H2 (3.214 ppm) and H3 (3.62 ppm) resonances of the α anomer. For the minor β anomer, only a weaker three-bond correlation between carbon-bound H3(3.582 ppm) and ^{15}N was observed. The IMPACT-HNMBC spectrum in Figure 2.9 was acquired after adjusting the pH to 7.48 in aqueous solution and exchanging the solvent for 100% D_2O , however the choice of solution pH should not affect this spectrum as the carbon-bound proton intensities and chemical shifts are pH-independent between

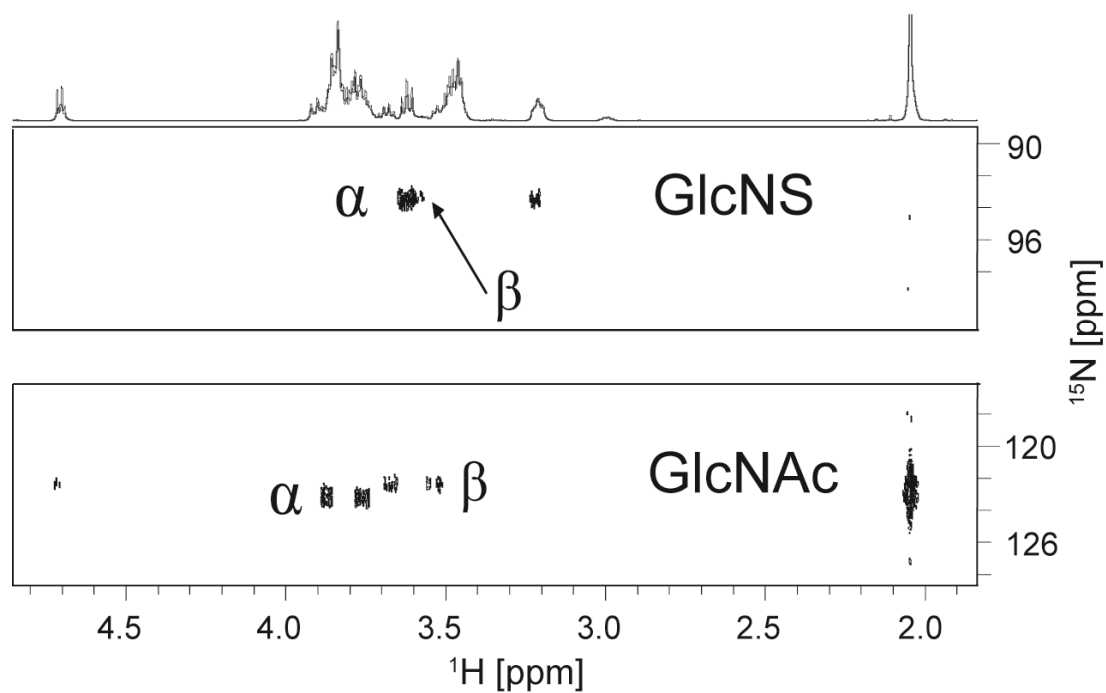


Figure 2.9. [^1H , ^{15}N] IMPACT-HNMBBC spectrum of 100 mM GlcNS and 100 mM GlcNAc in D_2O solution at 25 °C.

pH 2 and 12 after allowing for anomeric equilibration. While the IMPACT-HNMBC spectrum in Figure 2.9 shows correlations to the NH groups of each anomer of both monosaccharides, it provides fewer correlations to carbon-bound protons than in the HSQC-TOCSY spectrum in Figure 2.6.

While monosaccharides represent the simplest example to demonstrate the performance of the IMPACT-HNMBC experiment, similar results have been previously published in our report on the development of the [^1H , ^{15}N] IMPACT-HNMBC pulse sequence.¹⁵ To push the boundaries of this experiment, the spectra for the Arixtra oligosaccharide (Figure 2.5A) was measured using the [^1H , ^{15}N] IMPACT-HNMBC pulse sequence. Arixtra was chosen due to its availability and structural complexity, having three GlcNS residues in different local chemical environments. Figure 2.10 shows the IMPACT-HNMBC spectrum of a 40 mM Arixtra solution in D_2O . Based on the previous experiments with GlcNS, cross peaks between the H2 and H3 resonances and the sulfamate nitrogen are expected for each GlcNS residue. Surprisingly, only four cross peaks are observed in this spectrum even after 24 hr of data acquisition. All of the expected heteronuclear correlations were observed only for the non-reducing end GlcNS(I) residue. The GlcNS(I) H3 showed the strongest cross peak to the sulfamate ^{15}N , while H2 showed a much weaker cross peak. For the internal GlcNS(III) residue, a single weak correlation between H2 and the adjacent nitrogen was observed above the noise

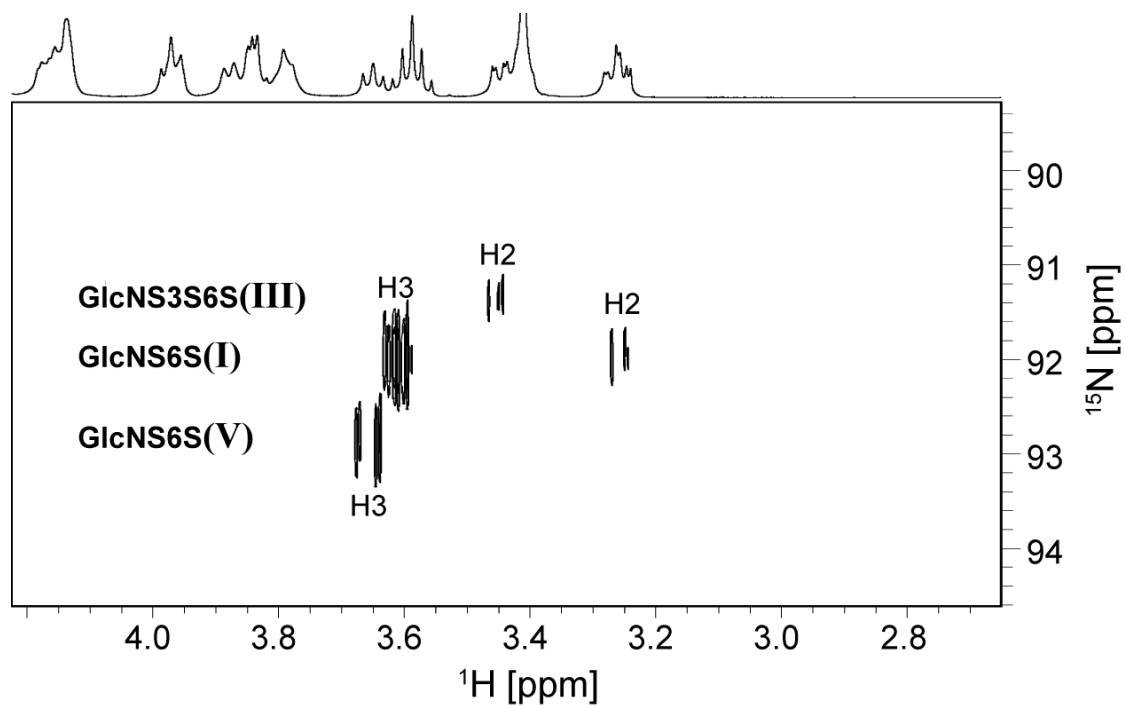


Figure 2.10. [^1H , ^{15}N] IMPACT-HNMBBC spectrum of Arixtra in D_2O analyzed at 25 °C in 300 μL using a 5 mm D_2O -matched Shigemi NMR tube.

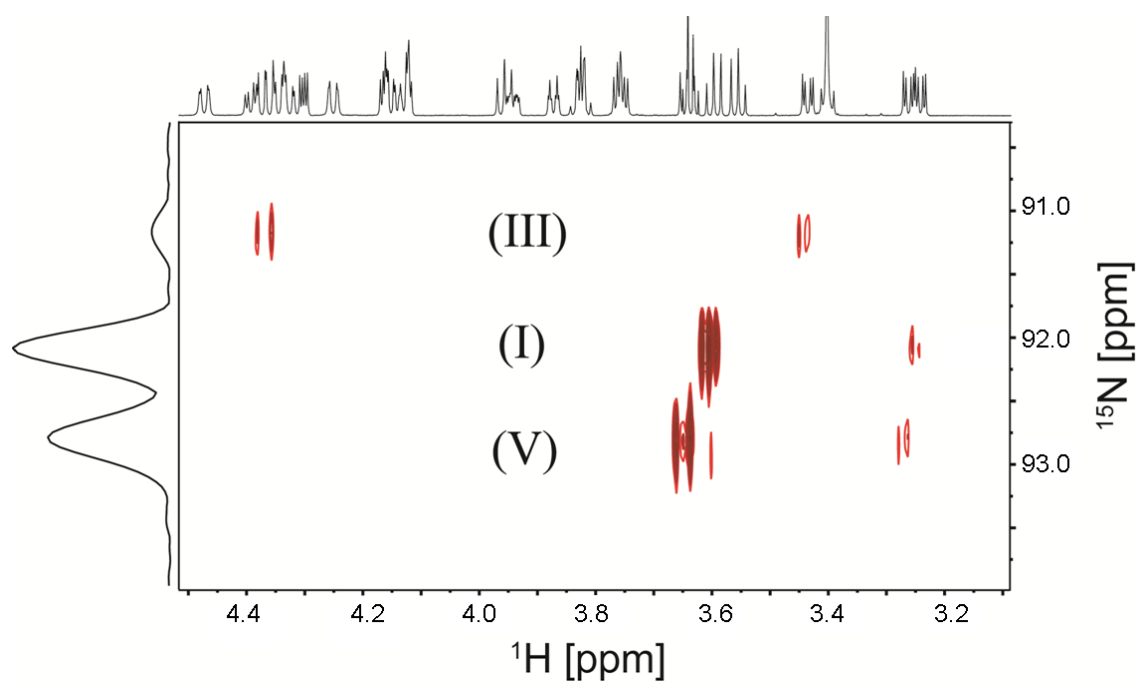


Figure 2.11. [^1H , ^{15}N] IMPACT-HNMB of Arixtra measured at 800 MHz using a cold probe.

floor. In the case of the GlcNS(V) residue, only a single cross peak between the H3 and the ^{15}N resonances could be detected. A limitation in performing this measurement is that an average long-range coupling constant must be selected, in this case 3 Hz. The different GlcNS residues may have slightly different multiple bond J couplings, however, performing the experiment using J values of 4 Hz and 5 Hz did not improve the spectral quality.

The main difficulty in the HNMBC experiment, especially for compounds with ^{15}N at natural abundance, resides in its inherent low sensitivity due to the small values of the long-range coupling constants and the ^{15}N line widths of these compounds. Repeating this experiment on a higher field magnet (800 MHz) with a more sensitive cryoprobe yields an additional correlation from the GlcNS(III) sulfamate nitrogen to H3, reflecting the 3-O sulfation at this position, as shown in Figure 2.11. Even with the use of a higher field magnet and cold probe, the quality of the information obtained from this spectrum is not significantly more expansive.

While both the [^1H , ^{15}N] HSQC-TOCSY and the [^1H , ^{15}N] IMPACT-HNMBC experiments possess the ability to detect different anomers of GlcNS and GlcNAc, only the [^1H , ^{15}N] IMPACT-HNMBC pulse sequence is able to measure the ^{15}N chemical shifts of the unsubstituted amino group of glucosamine (GlcNH). Despite the rapid exchange of the amino group protons with the aqueous solvents, the [^1H , ^{15}N] IMPACT-HNMBC spectrum detects ^{15}N indirectly through long-range correlations to carbon-bound ring protons as illustrated in Figure 2.12 for GlcNH(3S) in D_2O at pD 7. The resonances of both the α (60%) and β (40%) anomers of GlcN(3S) are observed and are resolved in the

^{15}N dimension. The ^{15}N and ^1H chemical shifts determined for GlcNS(3S) are summarized in Table 2.2. In the HNMBC spectrum in Figure 2.12, a strong cross peak between H3 and ^{15}N was detected for the α -anomer. Interestingly, in the case of the β -anomer, both H1 and H3 showed intense correlations to the amino ^{15}N . It is also interesting to note the effect of the 3-*O*-sulfo substitution on ^{15}N chemical shift. Limtiaco et al. reported that sulfate substitution at the 6-*O* position of GlcN had only a nominal impact on the ^{15}N chemical shift compared to unsulfonated GlcN.¹⁵ This contrasts the effect of substitution at the 3-*O* position, which results in a shift downfield from the GlcN ^{15}N chemical shifts of 32.49 ppm (α) and 29.93 ppm (β) to 35.05 ppm (α) and 33.56 ppm (β) in GlcN(3S). This information will likely be useful in aiding in the interpretation of [^1H - ^{15}N] HSQC spectra measured for larger oligosaccharides or intact GAG polymers with ^{15}N -enrichment isolated from cell-culture.⁹

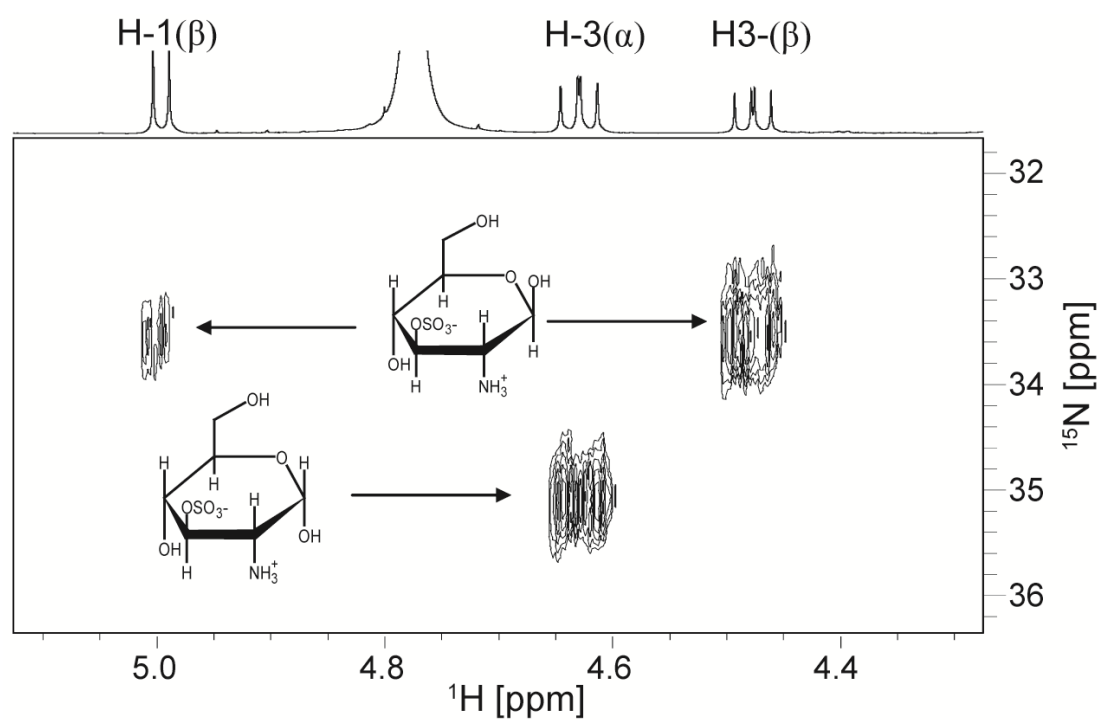


Figure 2.12 [^1H , ^{15}N] IMPACT-HMBC spectrum of 150 mM GlcN(3S) in 100% D_2O with 10 mM phosphate at pH 7.0 and 25 °C.

Table 2.2. Summary of the ^1H and ^{15}N NMR chemical shifts (in ppm) measured using the [^1H , ^{15}N] IMPACT-HNMBC experiment for the compounds studied. Chemical shifts measured using one-dimensional ^1H NMR spectra are reported to three decimal places. Chemical shifts determined from 2D spectra are reported to two decimal places due to the poorer digital resolution of these measurements.

IMPACT-HNMBC	^{15}N	H1	H2	H3
GlcNAc(α)	123.20		3.875	3.764
GlcNAc(β)	122.40	4.719	3.670	3.539
GlcNS(α)	93.52		3.214	3.619
GlcNS(β)	93.06			3.582
Arixtra GlcNS(I)	91.90		3.26	3.602
Arixtra GlcNS(III)	91.35		3.442	
Arixtra GlcNS(V)	92.85			3.649
GlcNH(3S)(α)	35.05			4.619
GlcNH(3S)(β)	33.56	4.986		4.447

2.4 Conclusions

This work reflects the first published report of the detection of the sulfamate groups of GlcNS by ^1H NMR. This accomplishment was confirmed through analysis with the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC experiment which selectively measures nitrogen bound protons. The ^{15}N chemical shift of the monosaccharide GlcNS was consistent with that published previously by our group through indirect detection using the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ IMPACT-HNMBC pulse sequence. The ^1H NMR spectra of GlcNS measured as a function of pH show that the exchange of the sulfamate groups with the aqueous solvent is minimized in the pH range of 6.67 to 8.54. Having the ability to consistently detect sulfamate groups of GlcNS isomers by ^1H and ^{15}N NMR provides the means, for the first time, to analyze heparin and HS-derived, oligosaccharides and holds the potential for characterization of intact GAGs and complex mixtures such as LMWH, as discussed in Chapter 4.

Additionally, this work demonstrates the utility of the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC-TOCSY pulse sequence for determining ^{15}N chemical shifts and their intra-ring aliphatic connectivities allowing complete elucidation of GlcNS and GlcNAc spin systems. When solution conditions can be optimized for the measurement of single quantum NH correlations, the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC-TOCSY experiment is superior to the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ IMPACT-HNMBC pulse sequence, providing both better sensitivity and more complete long-range correlations which are essential for the structure determination of GAG-derived samples. However, the IMPACT-HNMBC experiment is able to observe *N*-unsubstituted GlcN residues whose protons exchange too rapidly with the aqueous solvent to be detected through the $^1J_{(\text{NH})}$ coupled protons. While each of these

experiments has their own advantages and pitfalls, it is important to note that they remain complementary to each other and orthogonal to other existing heteronuclear NMR methods.

Having the ability to measure the ^1H and ^{15}N chemical shifts of the sulfamate groups of the GlcNS residues found in heparin and HS provides a new avenue to study this class of molecules. The spectroscopic study of Arixtra provided a wealth of information regarding what information we can expect to obtain from studying sulfamate groups of GlcNS in oligosaccharides beyond the fundamental building blocks. The ^1H NMR spectrum of Arixtra tells us that the sulfamate resonances of GlcNS can have variation in chemical shifts as well as line widths, presumably based on elements of primary or secondary structure; a topic that will be explored in greater detail in Chapter 3. Additionally, the observation of discrete [^1H , ^{15}N] correlations for each GlcNS residue in the [^1H , ^{15}N] HSQC spectrum of Arixtra suggest that these chemical shifts are sensitive to elements of structure which has the potential to extend to larger oligomers. Chapters 3 and 4 will discuss the application of line width and chemical shift analysis of the sulfamate groups of GlcNS residues and their potential for the characterization of heparin HS and their derivatives.

2.5 References

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CHAPTER THREE

Sulfamate proton solvent exchange in heparin oligosaccharides – Evidence for a persistent hydrogen bond in the antithrombin-binding pentasaccharide Arixtra

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Abstract: In this chapter proton NMR resonance line shape analysis is used to explore the solvent exchange properties of the sulfamate NH groups within a heparin-related mono-, di-, tetra- and pentasaccharide as a function of pH and temperature. The results of these experiments identified a persistent hydrogen bond within the Arixtra (Fondaparinux sodium) pentasaccharide between the internal glucosamine sulfamate NH and the adjacent 3-*O*-sulfo group. This discovery provides new insights into the solution structure

of the Artixtra pentasaccharide and suggests that 3-*O*-sulfation of the heparin *N*-sulfoglucosamine residues pre-organizes the secondary structure in a way that facilitates binding to antithrombin-III. NMR studies of the *N*-sulfoglucosamine NH groups can provide important information about heparin structure complementary to that available from NMR spectral analysis of the carbon-bound protons.

3.1 Introduction

The molecular-level characterization of the glycosaminoglycans (GAGs) heparin and heparan sulfate is of critical importance to advancing our understanding of the relationship between elements of structure and the myriad of biological processes mediated by these biopolymers through their interactions with proteins.¹ It is becoming increasingly clear that GAGs may contain regular elements of structure,^{2, 3} and that in many cases these structural features regulate diverse biological processes through specific GAG-protein interactions.⁴

The major heparin disaccharide sequence is the trisulfated L-IdoA(2S)-D-GlcNS(6S), which comprises over 70% of heparin isolated from porcine intestinal mucosa, however there is significant structural variability along the heparin chain that is important for its biological functions.⁵ For example, Arixtra (Fondaparinux sodium) was designed to mimic the heparin pentasaccharide responsible for binding to AT-III, which is found on average in only one-third of the heparin chains.⁶ Among the structural elements of Arixtra important for high affinity binding are the unsulfated GlcA residue, and the 3-*O*-sulfo group of the center GlcNS residue. Atha et al. reported a 1000-fold

decrease in the affinity of the native pentasaccharide for antithrombin III when the 3-*O*-sulfate group was removed.⁷ This 3-*O*-sulfo group provides only one of Arixtra's ten negative charges suggesting that the contribution of this moiety to binding is more than electrostatic.^{8,9}

The structures of the compounds investigated in this chapter are shown in Figure 3.1. The monosaccharide and the reducing-end GlcNS residues of the di- and tetrasaccharide are present in both the α and β anomeric forms. The structures of the di- and tetrasaccharide shown in Figure 3.1 also contain a double bond in the non-reducing end uronic acid residue that was introduced by the heparinase enzyme used in the depolymerization reaction employed prior to isolation of these compounds.¹⁰

In addition to chemical shift assignments, the solvent exchange kinetics of NH protons can potentially provide important insights into oligosaccharide structure.¹¹ Line shape analysis of the NMR resonances of exchanging protons (e.g. the hydroxyl and amide groups commonly found in carbohydrates) can provide rich structural information. Herein, we present the first detailed characterization of the pH- and temperature-dependence of sulfamate proton-solvent exchange for a heparin mono-, di- and tetrasaccharide, as well as the synthetic Arixtra pentasaccharide.

This study builds on the well-established literature on peptide and protein amide proton exchange,¹² which is now a powerful tool for probing hydrogen bonding,¹³ secondary structure,¹⁴⁻¹⁶ and folding.¹⁷⁻¹⁹ As in proteins, sulfamate NH temperature coefficients and experimentally-determined barriers for solvent exchange (assessed by measuring the resonance line width as a function of temperature)²⁰ can reflect the extent

to which sulfamate protons are protected from exchange through hydrogen-bonding. Because the heparin sulfamate protons were previously believed to exchange too fast to be detected by NMR, their potential to report on hydrogen-bond formation in solution has not been exploited, although the growing number of crystal structures of heparin oligosaccharide-protein complexes suggest that the *N*-sulfo groups are structurally important.²¹⁻²⁶ Our experimental results provide the first evidence for a persistent hydrogen bond involving an Arixtra sulfamate proton, a finding that is further substantiated through molecular dynamics simulation.

3.2 Methods and Materials

3.2.1. Materials and reagents. *N*-sulfoglucosamine and chemical shift reference 2,2-dimethyl-2-silapentane-5-sulfonate-*d*₆ sodium salt (DSS) were purchased from the Sigma Chemical Company (St. Louis, MO). Dibasic sodium phosphate heptahydrate and sodium bicarbonate were purchased from Fisher Scientific (Pittsburgh, PA). The disaccharide Δ UA(2S)-GlcNS(6S) was purchased from V-labs (Covington, LA). Arixtra was obtained through the University Pharmacy and Department of Pharmacy Administration of Semmelweis University, formulated as prefilled syringes. The Arixtra solutions were pooled and desalted using a 1.6 x 70 cm Sephadex G10 superfine column (GE Healthcare) with a flow rate of 0.15 mL/min using HPLC grade water as the eluent. The hexasulfated heparin-derived tetrasaccharide Δ UA(2S)-GlcNS(6S)-IdoA2S-GlcNS(6S) was isolated as previously described by Limtiaco et al.²⁷

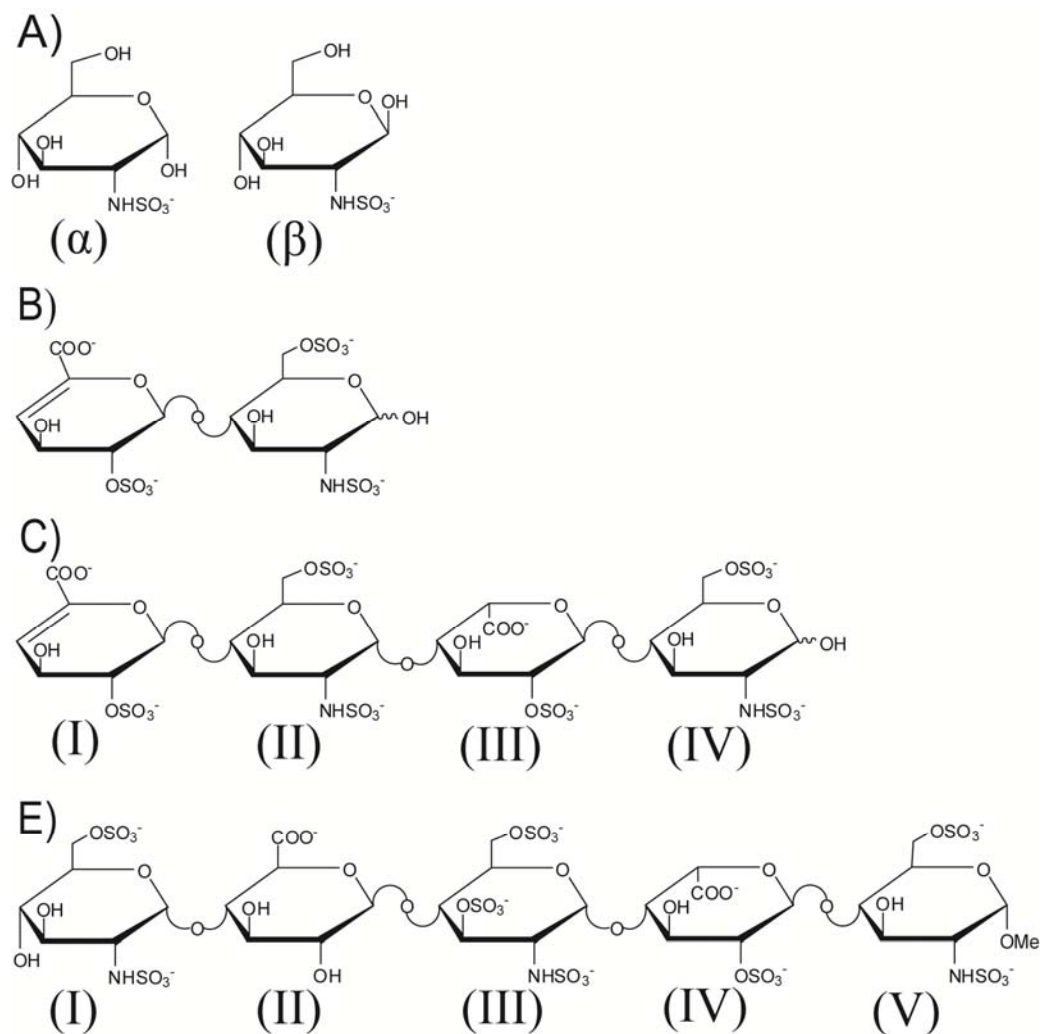


Figure 3.1. Structures of compounds used in this study. A) *N*-sulfoglucosamine showing the structures of both the α and β anomeric forms, B) heparin disaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})$ where ΔUA indicates the unsaturated uronic acid residue introduced by heparin lyase cleavage, C) heparin tetrasaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-IdoA}(2\text{S})\text{-GlcNS}(6\text{S})$, D) the Arixtra pentasaccharide. For the structures in (B) and (C) the presence of the α and β anomers is indicated by a wavy bond for the reducing end anomeric OH group.

Solution pH measurements were performed using a Fisher Scientific AB15 pH meter equipped with a double junction Ag/AgCl micro pH electrode (Thermo Scientific, Beverly, MA) calibrated using pH buffers at 4.00, 7.00 and 10.00 (Fisher Scientific, Pittsburgh, PA) at 25° C. Saccharide solutions used for the NMR pH titrations were prepared in 90% H₂O/10% D₂O containing 25 mM phosphate, 25 mM bicarbonate and 3 mM DSS, with the exception of the GlcNS sample for which no bicarbonate was added. Adjustments to solution pH were made using phosphoric acid (Fisher Scientific, Pittsburgh, PA) and NaOD (Cambridge Isotopes Andover, MA) in Burdick & Jackson HPLC grade water (Honeywell, Morristown, NJ) and are reported without compensation for the deuterium isotope effect. Solutions used for measurement of spectra as a function of temperature were prepared in 90% H₂O/10% D₂O and 25 mM phosphate buffer containing 3 mM DSS adjusted to the pH optimum for each saccharide. All reported solution pH values were measured at 298 K.

3.2.2. NMR parameters. All ¹H NMR spectra were measured using a Bruker Avance spectrometer operating at ¹H frequency of 600.13 MHz using a triple resonance inverse (TXI) probe. Saccharide ¹H NMR spectra were measured as a function of pH and temperature using WATERGATE-W5 solvent suppression (Bruker pulse program *zgpgw5*) to attenuate the water resonance.²⁸ Spectra were referenced to DSS (0 ppm) and processed with zero-filling to 65536 points and multiplication by an exponential function equivalent to 0.3 Hz line broadening. Sample temperatures were calibrated using an external low temperature methanol standard from Cambridge Isotope Labs (Andover, MA) and calculated using the Van Geet equation.²⁹ At least 10 min were allowed for

sample thermal equilibration prior to spectral measurements. Temperature coefficients were determined from linear regression of NH chemical shift plotted as a function of temperature. The solution pH values for NMR spectra acquired as a function of temperature were measured at 25 °C and no attempt was made to correct for the temperature dependence of the solution pH.

The GlcNS solution was prepared at a concentration of 5 mM and used for both pH and temperature-dependent experiments. Temperature-dependent spectra were acquired at pH 7.4. The pH and temperature dependant spectra measured for GlcNS were collected into 65536 complex points using 64 scans and 16 dummy scans with a spectral window of 11 ppm. A 3.0 s relaxation delay was used along with a 200 us delay for WATERGATE solvent suppression. For the disaccharide Δ UA(2S)-GlcNS(6S), NMR spectra measured as a function of pH were collected and processed identically to those for GlcNS except that the sample concentration was 0.6 mM, 256 scans were collected, and a relaxation delay of 2.0 was used. Temperature-dependent measurements of the disaccharide were made at a concentration of 5.6 mM adjusted to pH 8.23. Spectra of a 2 mM Arixtra solution were measured as a function of pH as described for GlcNS except that 160 scans were collected and a relaxation delay of 2.0 s was used. Temperature-dependent spectra of Arixtra were collected as described for GlcNS using a 3 mM sample adjusted to either pH 8.26 or 7.43.

The WATERGATE-TOCSY spectrum used for assignment of the sulfamate protons of the tetrasaccharide Δ UA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S) was acquired at 22 °C with the Bruker pulse program *mlevgpphw5* using States-TPPI. The sample

concentration was 4 mM in 20 mM phosphate buffer prepared in 90% H₂O/10% D₂O and adjusted to pH 8.4. The spectrum was acquired into 4096 complex points in t_2 with 40 scans coadded at each of 512 t_1 increments. A 2 s relaxation delay was used along with a 200 μ s delay for solvent suppression. A spectral window of 3600 Hz was used in both dimensions with a mixing time of 120 ms. The spectrum was zero filled to 4096 x 2048 data points and apodized in both dimensions using a $\cos^2$ window function prior to Fourier transformation.

Resonance line widths were determined by nonlinear least squares fitting of a Lorentzian peak shape to the experimental spectra using a simulation program written in the Mathematica³⁰ programming environment.³¹ The details regarding the fitting procedure can be found in Appendix I and a representative example is shown in Figure 3.2. All ¹H spectra subjected to line shape analysis were processed with 0.3 Hz line broadening. Input parameters used in fitting were the resonance positions, amplitude, line width and ¹J-coupling values. Starting parameters were estimated from scale readings and adjusted manually. Spectra measured as a function of temperature were simulated using fixed ¹J values determined from the spectral simulation at the lowest temperature measured where the sulfamate resonances are sharpest. Due to the broader resonances in the spectra measured at 25 and 37 °C, the ¹J values were included as a fitting parameter in the simulation of spectra acquired as a function of pH. Spectra in which the sulfamate resonances were significantly overlapped with carbon bound proton resonances were not used for subsequent calculations because they produced fits with poor reliability.

3.2.3 Molecular dynamics simulation. The coordinates for the starting structure of Arixtra were extracted from the crystal structure of Antithrombin-S195A factor Xa (PDB ID 2GD4, pentasaccharide coordinates from residue ID 901).³² AmberTools 1.5 *xleap* module was used to generate the parameter/topology and coordinate files for the molecular dynamics simulation using GLYCAM-06g parameters for Arixtra.^{33, 34} GlcNS residues were modified from standard GLYCAM GlcNAc residues by removing the acetyl groups and adjusting the partial charge on the nitrogen to give the completed sulfamate group a -1 charge overall. The *xleap* module was also used to add ten sodium ions (ff99SB parameters) to neutralize the system charge (*addions* function) and to construct a cubic water box with each face a distance of 12 Å from the solute (3493 TIP3P water molecules).^{34, 35} All simulation steps, including minimization, heating, equilibration, and the production run were calculated using NAMD 2.8 (NAMD was developed by the Theoretical and Computational Biophysics Group in the Beckman Institute for Advanced Science and Technology at the University of Illinois at Urbana-Champaign).³⁶ Conjugate gradient minimization was carried out separately on the solvent, solute, and finally the whole system to remove possible bad contacts. Periodic boundary conditions were applied throughout. The particle mesh Ewald algorithm was used to treat long range electrostatic interactions with the grid spacing set to 1 Å.³⁷ Non-bonded interactions were cutoff at 12 Å and a smooth switching function applied at 10 Å. The VDW and electrostatic 1-4 scaling factors (SCNB and SCEE respectively) were set to 1 for consistency with GLYCAM-06 parameterization.³³ The SETTLE algorithm was

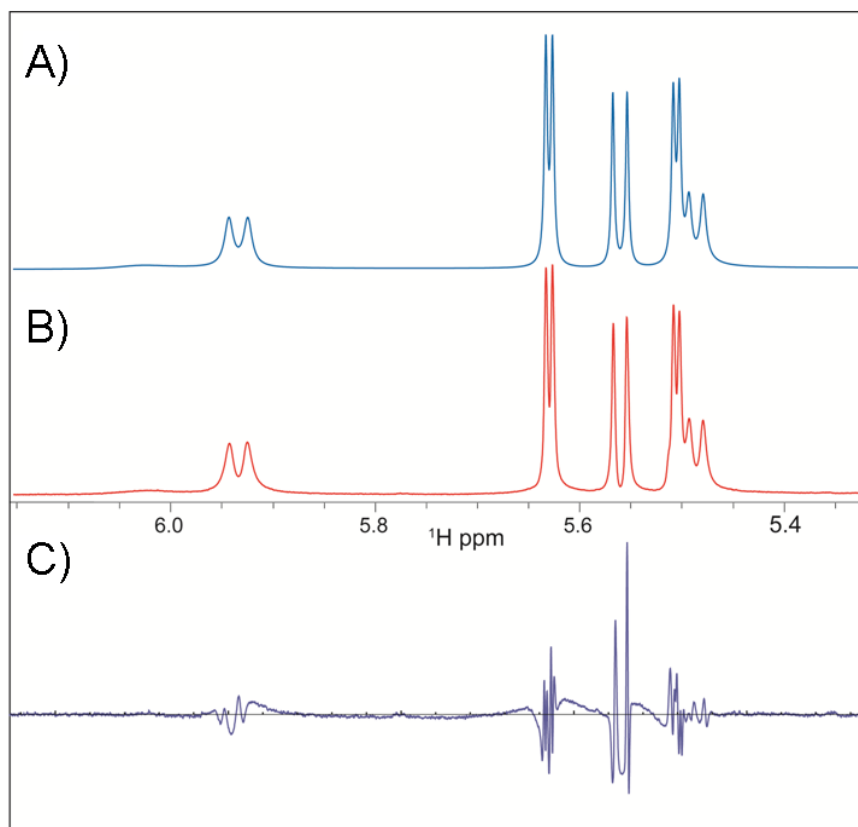


Figure 3.2. Example simulation of an Arixtra spectrum measured at 283 K. A) Simulated spectrum, B) measured spectrum, C) residual (experimental – simulated) spectrum plotted at a 20-fold expansion of the vertical scale.

used to maintain rigid bonds for water only.³⁸ Simulation time steps were 1 fs and the trajectory output was saved every 100 fs. The system was slowly heated under NVE conditions from 0 K to 300 K over 398 ps including a 98 ps hold at the target temperature. A 1 ns equilibration run preceded the 5 ns production run and both were calculated in the constant NPT ensemble (300 K and 1 atm). The temperature was held constant using the Langevin thermostat with a damping coefficient of 5 ps^{-1} , and the pressure was maintained using NAMD's Nosé-Hoover Langevin piston method. VMD 1.9 was used for the visualization of all trajectories.³⁹ Post-trajectory calculations of dihedral angles, interatomic distances, average structures, and hydrogen bonding were all accomplished using AmberTools 1.5 *ptraj* module.³⁴ Hydrogen bonding parameters included a heavy atom cutoff distance of 3.5 Å and an angle cutoff of 120°.

3.3. Results and Discussion

A molecule involved in an equilibrium involving chemically distinct sites can give rise to an NMR spectrum that differs in its appearance depending on the rate of exchange.^{40, 41} Common examples include protonation and complexation equilibria or rotational isomerism for example, the interconversion of the cis and trans isomers of proline-containing peptides.⁴⁰⁻⁴² If the exchange is slow on the NMR timescale, then separate sharp resonances are observed for the nuclei in each site. If the rate of exchange is fast on the NMR timescale, the spectrum is simplified and a single sharp resonance is observed for each nucleus at a chemical shift determined by the population-weighted average of the chemical shifts of each site in the absence of exchange. Whether an

equilibrium lies in the fast or slow exchange limit depends both on the exchange rate and the difference in chemical shift in Hz of the nuclei in the two exchanging sites. Since this chemical shift difference depends on the strength of the applied magnetic field, a spectrum may be in fast exchange at a lower field, but slow exchange when a higher field magnet is employed. Similarly, an exchanging system may give rise to a ^1H NMR spectrum in fast exchange due to the relatively narrow chemical shift range over which protons are detected, but be in slow exchange on the ^{19}F NMR time scale because of the much larger chemical shift dispersion of the ^{19}F nuclei.

Systems that lie between the fast and slow exchange limits are said to be in the intermediate exchange regime. In intermediate exchange, the observed line width is inversely proportional to the lifetime of the exchanging nuclei in each state and therefore is proportional to the exchange rate. In this chapter the resonance line width of the sulfamate nitrogen-bound protons are used to indicate rate by which they exchange with the water protons of the bulk solvent. Line shapes are determined through spectral simulation as illustrated by Figure 3.2.

3.3.1. pH dependence of the sulfamate ^1H resonance line width. Our initial experiments involved evaluation of the effect of pH on the solvent exchange rate using line shape analysis of the sulfamate ^1H NMR resonances in spectra measured with suppression of the water resonance using the WATERGATE–W5 pulse sequence.²⁸ At sites that participate in chemical exchange, the NMR line width shows an additional broadening that is initially proportional to the rate of exchange.²⁰ At first glance, one might expect that the minimum in the sulfamate ^1H -solvent exchange rate would occur in

acidic solution, as is common for amide protons.^{12, 43} Instead, the pH minimum for the exchange reaction occurs in slightly basic solution, as shown in Figure 3.3 for the GlcNS α and β anomers, a heparin disaccharide, and the three NHSO_3^- groups of Arixtra.

Figure 3.3A compares the effect of pH on the NH line width of the α and β anomeric forms of the GlcNS monosaccharide at 25 °C. Although the two GlcNS anomers interconvert through mutarotation, exchange across that equilibrium is much slower than the rate of NH solvent exchange and can be neglected in the line shape analysis. As seen in Figure 3.3A, the solution pH that minimizes the exchange rate of the α anomer occurs at 7.85 while the minimum for the β anomer is lower, at pH 7.20. In Chapter 2, (Figure 2.4) the [^1H , ^{15}N] HSQC spectrum of GlcNS was measured at pH 7.50 which corresponds to the center point for the optimum pH of each anomer. The NH of the GlcNS α -anomer also appears to be stabilized with respect to solvent exchange as the resonance line width at the pH minimum (9.73 Hz) is significantly lower than the minimum observed for the β anomer (26.30 Hz). This suggests that the axial OH group of the α anomer reduces the rate of NH exchange with water compared to the β anomer. Compared with the GlcNS monosaccharide, the more basic pH (8.21) measured for the minimum resonance line width (9.54 Hz) of the disaccharide α anomer (Figure 3.3B) may be attributed to the greater overall negative charge of the disaccharide as discussed in section 2.3.1.

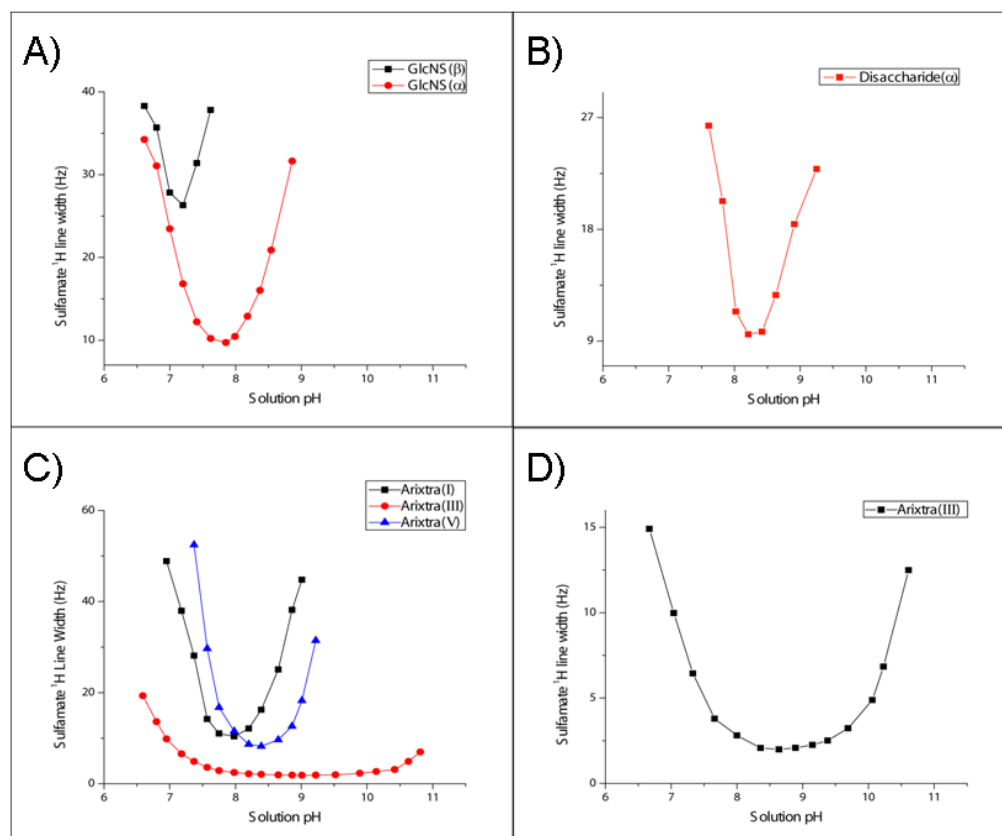


Figure 3.3. Variation of sulfamate ^1H resonance line width as a function of pH for A) the α and β anomers of GlcNS, B) the disaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})$ α anomer, C) Arixtra measured at 25 °C, D) Arixtra GlcNS(III) measured at 37 °C.

The Arixtra pentasaccharide is interesting because it contains three structurally unique GlcNS residues (Figure 3.1E), giving rise to three well-resolved sulfamate ^1H NMR resonances.⁹ Figure 3.3C shows the pH dependence of the Arixtra sulfamate protons at 25 °C. The Arixtra GlcNS(I) at the non-reducing end of the pentasaccharide is 6-*O*-sulfated and is coupled via the glycosidic linkage to an unsulfated GlcA residue. The pH minimum for the GlcNS(I) NH proton is 7.98 giving a minimum line width of 10.38 Hz. The Arixtra reducing end GlcNS(V) residue is also 6-*O*-sulfated and is locked in a single anomeric configuration by the methyl glycoside. The curve obtained for the reducing end GlcNS(V) is similar to that of GlcNS(I), but is shifted slightly giving a pH minimum of 8.39 and a minimum line width of 8.23 Hz. The most interesting behavior in Figure 3.3C is observed for the central 3-*O*- and 6-*O*-sulfated GlcNS(III) residue of Arixtra. This sulfamate proton resonance is much sharper than the resonances of the other residues at all pH values examined and the range over which this resonance can be detected spans more than 4 pH units. We avoided using very basic conditions (i.e. pH > 10.8) in these experiments due to the potential for hydrolysis of the reducing end methyl glycoside. In the region from pH 8.65 – 9.52 in Figure 3.3C, the measured line width is near the limiting value, ~1.8 Hz. In this pH regime, the rate of solvent exchange is sufficiently slow that the resonance line width puts only an upper bound on the exchange rate. Measurements made as a function of pH at 25 °C were unsuccessful in identifying the pH minimum for the exchange of this sulfamate proton.

Repeating this experiment at 37 °C (Figure 3.3D) sufficiently increased the reaction rate to permit observation of a minimum in the solvent exchange reaction at pH 8.64 for the Arixtra GlcNS(III) sulfamate proton.

The more basic pH minimum determined for the GlcNS(III) NH might be attributed simply to its internal position embedded in the center of the pentasaccharide and the high local charge density. We attempted an experiment to measure the rate of exchange directly through addition of an aliquot of D₂O to an aqueous Arixtra solution, however, the reaction was complete by the time the first spectrum could be acquired, within 50 s.

Comparison of the structure of the saccharides studied (Figure 3.1) and the pH corresponding to the minimum in the sulfamate resonance line width (Figure 3.3) suggests that in slightly basic solution charge repulsion reduces the rate of hydroxide catalyzed solvent exchange. The GlcNS monosaccharide with line width minima of 7.85 and 7.20 for the α and β anomers, respectively, has a single sulfate group and a charge of -1. The minimum NH resonance line width of the disaccharide Δ UA(2S)-GlcNS(6S) α -anomer with a net charge of -4 occurs in more basic solution (8.21) (Figure 3.3B). This reinforces our observation, first discussed in section 2.3.1, that charge repulsion between the negatively charged saccharides and hydroxide ion reduces the exchange rate in basic solution. Additionally, we observe that buffer anions such as phosphate and carbonate do not appear to affect the sulfamate proton exchange rate, even though it is well-established that these anions can catalyze solvent exchange of hydroxyl protons in neutral saccharides.^{44, 45}

The Arixtra pentasaccharide provides the opportunity to further explore the relationship between saccharide structure and the kinetics of sulfamate proton solvent exchange. Arixtra has a high negative charge density due to its 8 sulfate groups and two carboxylate moieties, and its three GlcNS groups are in distinctly different local environments. As shown in Figure 3.3C, the NH resonance of the non-reducing end GlcNS(I) has a pH minimum of 7.98, less than the value determined for the disaccharide (8.21) and the Arixtra reducing end GlcNS(V) (8.39). The lower pH minimum observed for the solvent exchange of GlcNS(I) may be due to the lack of a flanking iduronic acid residue placing it in a region of lower net negative charge and reduced steric hindrance compared with the Arixtra GlcNS(V) residue. By comparison, the internal Arixtra 3-O-sulfated GlcNS(III) NH exchanges more slowly over a wide pH range. Such a radical difference in exchange behavior could also arise from the involvement of the GlcNS(III) NH in a persistent hydrogen bond, a possibility we explored further through variable temperature measurements.

3.3.2 Sulfamate NH temperature coefficients. Temperature coefficients ($\Delta\delta/\Delta T$) are also a well-established indicator of hydrogen bonding in peptides, proteins, and carbohydrates.^{44, 46-48} To probe the presence of hydrogen bonds temperature coefficients (Table 3.1) were measured for the sulfamate proton resonances of the compounds shown in Figure 3.1. Example temperature-dependent spectra for Arixtra shown in Figure 3.4 and graphical plots for all samples measured can be found in Figure 3.5. For GlcNS the temperature coefficients measured for sulfamate proton resonances of the α (-5.5 ppb/K) and β (-7.4 ppb/K) anomers reinforce the observation based on Figure 3.3A that the α

anomer sulfamate proton is partially shielded from solvent by the axially positioned anomeric OH group. Given the position of the GlcNS sulfamate moiety at the reducing end of the disaccharide Δ UA(2S)-GlcNS(6S), it is not surprising that the temperature coefficients determined for the disaccharide α (-5.6 ppb/K) and β (-6.9 ppb/K) anomer resonances closely resemble the values obtained for the monosaccharide GlcNS anomers.

The tetrasaccharide Δ UA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S) provides the opportunity to compare the temperature coefficients determined for the internal GlcNS(II) residue (-3.8 ppb/K) with that of the reducing end GlcNS(IV- α) (-5.3 ppb/K) and GlcNS(IV- β) (-6.7 ppb/K). The sulfamate group resonance assignments were made using TOCSY correlations (Figure 3.6). Although the values of the temperature coefficients measured for the tetrasaccharide reducing end α and β anomers are in good agreement with those determined for the mono- and disaccharide, the value of the internal GlcNS(II) temperature coefficient is significantly lower.

The most interesting results in Table 3.1 are the striking differences in the temperature dependence of the three sulfamate groups of Arixtra (Figure 3.5). The two NH resonances of the reducing (GlcNS(I)) and non-reducing (GlcNS(V)) ends of the pentasaccharide have identical temperature coefficients (-6.6 ppb/K), while the temperature coefficient of the center residue (GlcNS(III)) is much lower (-1.0 ppb/K). This low temperature coefficient for the NH resonance of the Arixtra 3-O- and 6-O-sulfated GlcNS(III) residue strongly supports the involvement of this proton in a hydrogen bond.

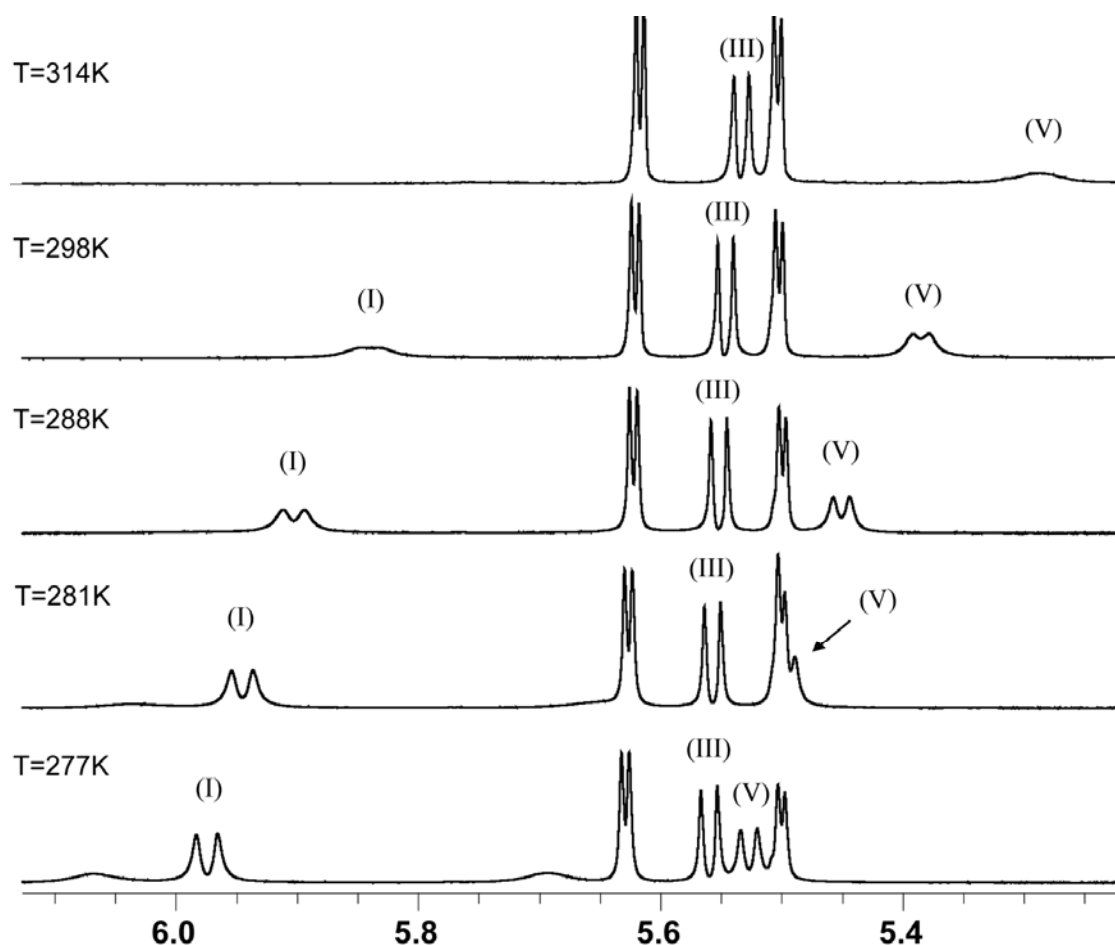


Figure 3.4. Selected ^1H NMR spectra measured for Arixtra as a function of temperature. The three NH resonances are labeled according to the positions of the GlcNS residues in the Arixtra structure (Figure 3.1). The doublets at 5.499 and 5.625 ppm are due to the carbon bound anomeric protons (H1) of GlcNS(III) and GlcNS(I), respectively.

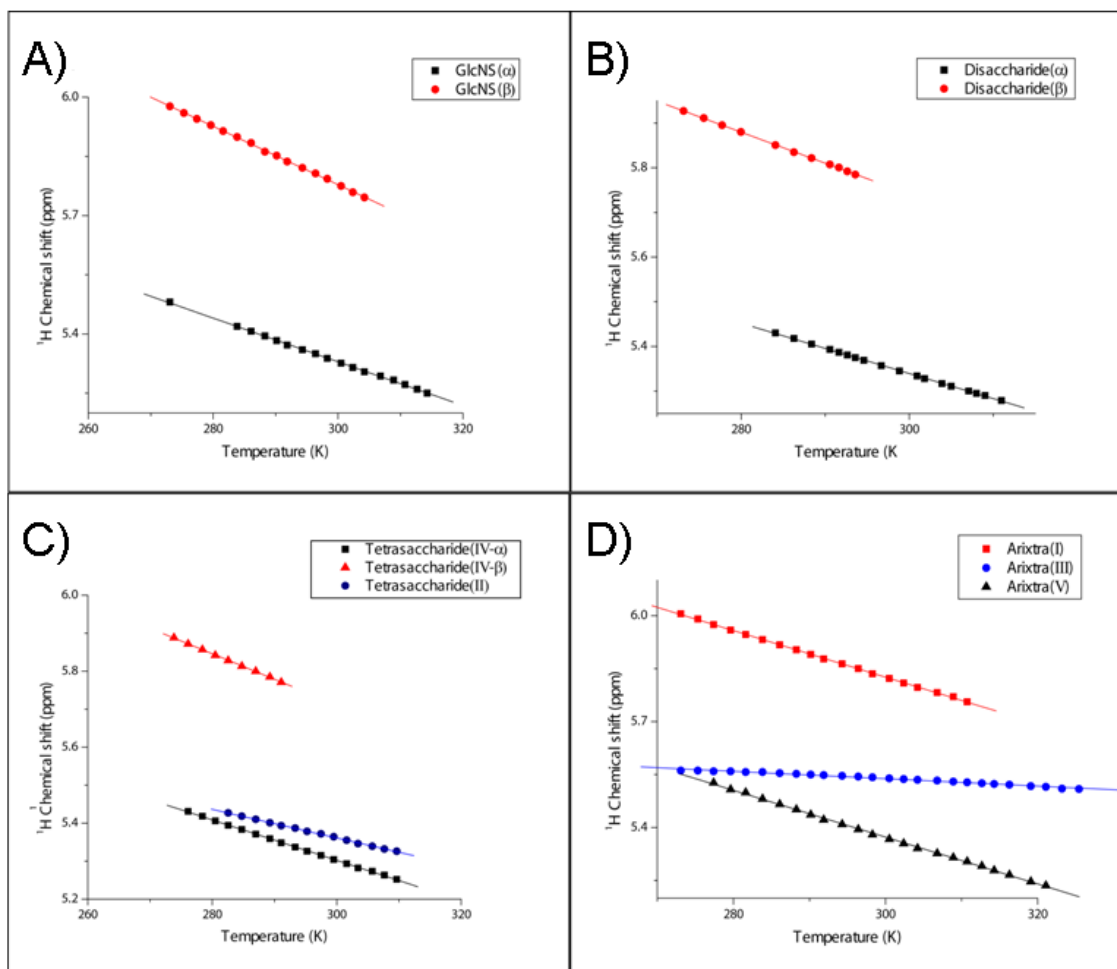


Figure 3.5. Graphs used to calculate ^1H temperature coefficients, $\Delta\delta/\Delta T$, for the sulfamate groups of A) GlcNS, B) heparin disaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})$, C) heparin tetrasaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-IdoA}(2\text{S})\text{-GlcNS}(6\text{S})$, D) Arixtra. Structures are given in Figure 3.1.

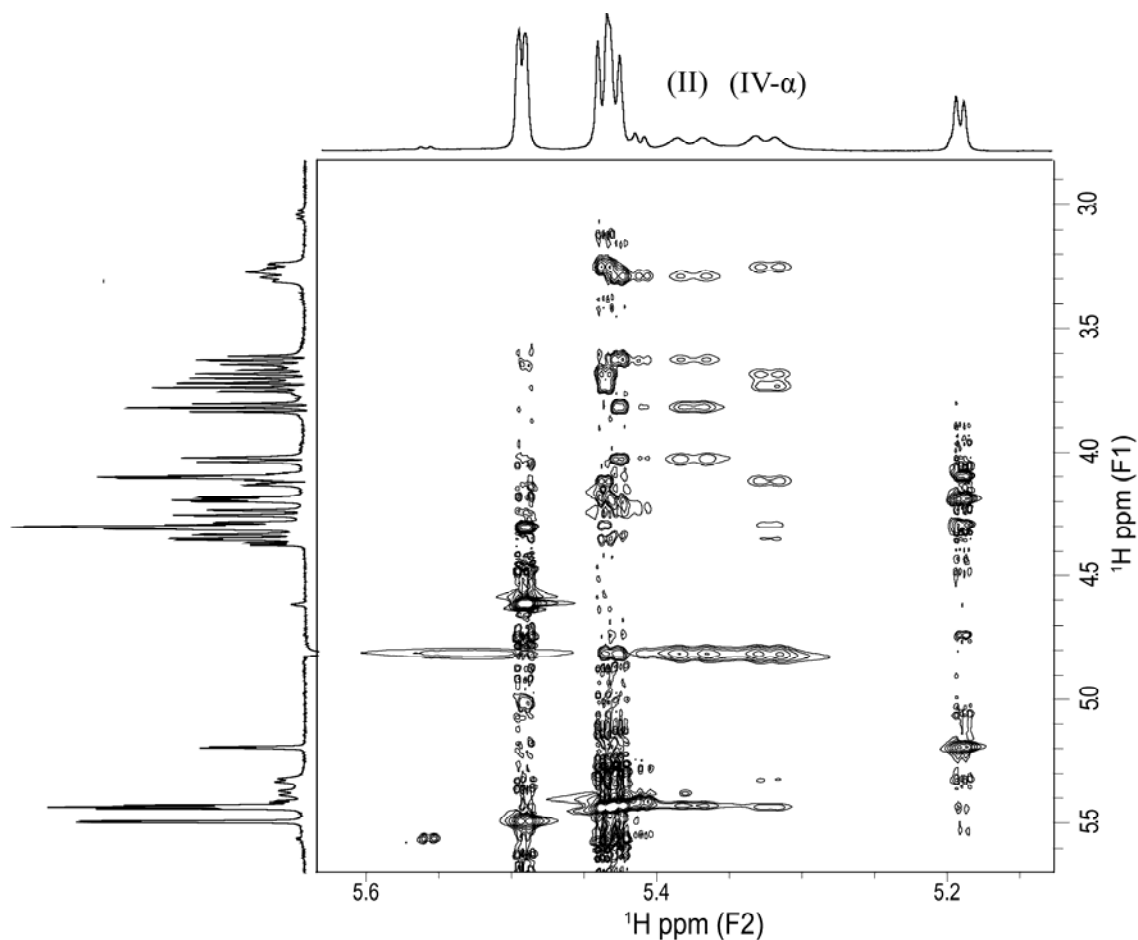


Figure 3.6. TOCSY-W5 spectrum used for the assignment of the sulfamate group ^1H NMR resonances of the heparin tetrasaccharide $\Delta\text{UA}(2\text{S})\text{-GlcNS}(6\text{S})\text{-IdoA}(2\text{S})\text{-GlcNS}(6\text{S})$ where F2 is plotted along the horizontal axis. The numbers refer to the sulfamate ^1H resonance of the internal (II) and reducing end (IV- α) GlcNS(6S) residues, as in Figure 3.1. The intense cross peaks at 4.816 ppm in F1 arise from chemical exchange of the sulfamate NH protons with water during the evolution period.

Table 3.1. GlcNS NH temperature coefficients measured in 90% H₂O/10% D₂O 20 mM phosphate buffer.

Saccharide	Sulfamate ¹ H NMR chemical shift (ppm)	Temperature coefficient (ppb/K)
GlcNS(α)	5.338 ^a	-5.5
GlcNS(β)	5.791 ^a	-7.4
Disaccharide GlcNS(α)	5.347 ^b	-5.6
Disaccharide GlcNS(β)	5.755 ^b	-6.9
Tetrasaccharide GlcNS(II)	5.372 ^c	-3.8
Tetrasaccharide GlcNS(IV- α)	5.315 ^c	-5.3
Tetrasaccharide GlcNS(IV- β)	5.733 ^d	-6.7
Arixtra GlcNS(I)	5.838 ^e	-6.6
Arixtra GlcNS(III)	5.542 ^e	-1.0
Arixtra GlcNS (V)	5.380 ^e	-6.6
a measured at 25.13 °C, pH 7.4		
b measured at 25.69 °C, pH 8.2		
c measured at 24.33 °C, pH 8.2		
d extrapolated to 24.33 °C, pH 8.2		
e measured at 25.13 °C, pH 8.2		

The temperature dependence of sulfamate proton resonance chemical shifts and line widths provides additional insights into the effects of structure on the kinetics of the exchange of the sulfamate protons with water. Similar temperature coefficients (Table 3.1, Figure 3.5) were determined for the reducing end GlcNS residues of the monosaccharide and the heparin-derived di- and tetrasaccharides. The temperature coefficients measured for the GlcNS α anomers were consistently lower than those measured for the β anomers suggesting a role for the axial anomeric OH group in partially protecting the α anomer sulfamate proton from solvent exchange, most likely through steric effects. The significantly lower temperature coefficient (-3.8 ppb/K) measured for the internal GlcNS(II) sulfamate proton of the tetrasaccharide suggests that it is more protected from solvent exchange than the GlcNS(IV) of the reducing-end. This reduced temperature coefficient may be attributed to both steric hindrance and charge repulsion of OH⁻ due to the net -8 charge of this tetrasaccharide, contributed by the 6 sulfate and 2 carboxylate groups. Alternatively this reduced temperature coefficient could reflect the involvement of the GlcNS(II) sulfamate group in a weak hydrogen bond.

As shown in Figure 3.4, the frequencies of the sulfamate protons of the two GlcNS residues at the reducing and non-reducing ends of the pentasaccharide shift towards the water resonance at similar rates (-6.6 ppb/K) and broaden as the temperature is raised, while the central GlcNS(III) resonance undergoes only a slight change in chemical shift and remains sharp even at 41 °C. The very low temperature coefficient determined for the central GlcNS(III) residue (-1.0 ppb/K) supports the results shown in

Figure 3.3 illustrating the effect of pH on the line width of this resonance, and taken together, provide evidence for a persistent hydrogen bond.

3.3.3. Calculation of the energy barrier for sulfamate proton solvent exchange. To complement the temperature coefficient measurements, temperature-dependent exchange broadening was evaluated for the NH resonances of GlcNS, the disaccharide ΔUA(2S)-GlcNS(6S) α anomer, and Arixtra. Spectra illustrating the temperature-dependent broadening of the three Arixtra sulfamate NH resonances are shown in Figure 3.4. Resonance line widths were determined as described in section 3.2.2, plotted vs. the sample temperature calibrated using an external methanol standard,²⁹ and the data fit using the Eyring–Polanyi equation (Eq. 3.1) to calculate the energy barrier, $\Delta G^\ddagger$, associated with proton exchange.^{20, 49, 50} In Eq. 3.1, k is the line width representing the relative exchange rate, k_B is the Boltzmann constant, and T is temperature in K.

$$k = \frac{k_B T e^{-\Delta G^\ddagger / RT}}{\pi h} \quad \text{Eq. 3.1}$$

The exponential fits obtained for the NH resonances of the compounds studied are provided in Figure 3.7. Line shape analysis was also performed for the sulfamate protons of the two GlcNS residues of the tetrasaccharide, but we were unable to arrive at suitable fits to the Eyring-Polyani equation due to overlap of the sulfamate ^1H resonances with resonances of the carbon-bound protons over a fairly wide temperature range.

Attempts to perform line shape analysis for the sulfamate resonance of the disaccharide β anomer failed due to its low intensity and because of spectral overlap with a ^{13}C satellite of the GlcNS H1 resonance.

The values of $\Delta G^\ddagger$ calculated for GlcNS, the disaccharide α anomer, and Arixtra are summarized in Table 3.2. The GlcNS(β) proton has the lowest barrier to exchange (15.1 ± 0.1 kcal/mol) and the largest temperature coefficient suggesting that for the compounds studied thus far, this proton exchanges most freely with the solvent. Similar $\Delta G^\ddagger$ values were obtained for the sulfamate protons of both the GlcNS (15.5 ± 0.1 kcal/mol) and disaccharide α anomers (15.6 ± 0.1 kcal/mol), again in good agreement with the temperature coefficients determined for these compounds. These results further suggest that the exchange rate of the GlcNS sulfamate proton is unaffected by the distorted conformation of the ΔUA ring in the disaccharide. The similar behavior of the sulfamate protons in these two compounds is chemically reasonable considering the similar environments of the GlcNS residues at the reducing end of the molecules.

The energy barriers to proton exchange calculated for the three GlcNS residues of Arixtra (Table 3.2) at pH 8.2 are very interesting. The GlcNS(I) residue at the non-reducing end of the pentasaccharide has the lowest barrier for exchange (15.1 ± 0.1 kcal/mol), similar to that calculated for the GlcNS(β) sulfamate proton. This residue is unusual in that it is not preceded by an uronic acid residue, and as a result may be more accessible to solvent. The value of $\Delta G^\ddagger$ (15.7 ± 0.1 kcal/mol) determined for the Arixtra reducing end GlcNS(V) is very close to the value of the α anomers of GlcNS and the

disaccharide. In contrast, the larger $\Delta G^\ddagger$ value measured for the central Arixtra GlcNS(III) sulfamate proton (18.1 ± 0.1 kcal/mol) is consistent with the additional energetic barrier to exchange expected if the proton were involved in a hydrogen bond, as suggested by the low temperature coefficient measured for this resonance.⁵¹⁻⁵³

The greater energy barrier for proton exchange with water provides further support for the involvement of the GlcNS(III) NH group in a hydrogen bond (Table 3.2). At pH 8.2, the $\Delta G^\ddagger$ value of GlcNS(III) is 2.4 kcal/mol greater than the value determined for the GlcNS(V) residue and 3.0 kcal/mol greater than the value determined for the non-reducing end GlcNS(I) sulfamate proton. The magnitude of the difference in this energy barrier ($\Delta\Delta G^\ddagger$) is consistent with what has been reported previously for amide proton hydrogen bonds.^{31, 52, 53} Line shape analysis performed at the more physiologically-relevant pH of 7.4 gives a $\Delta G^\ddagger$ for GlcNS(III) that is 1.8 kcal/mol greater than that measured for GlcNS(I), suggesting that the hydrogen bond may be involved in stabilizing the solution structure of Arixtra under conditions relevant to its pharmacological action.

3.4.4. Molecular dynamics simulation. While previous literature reports presenting experimental evidence for a structural hydrogen bond involving the GlcNS(3S)(6S) sulfamate proton in solution are lacking, a computational study of the central disaccharide of Arixtra reported an H-O distance of $1.92 \text{ \AA}$ between the NH and 3-O-sulfo oxygen, indicative of a hydrogen bond.⁵⁴ To better understand the role of sulfamate group hydrogen bonding in the Arixtra and illuminate our experimental results, a preliminary molecular dynamics simulation of the full pentasaccharide was performed

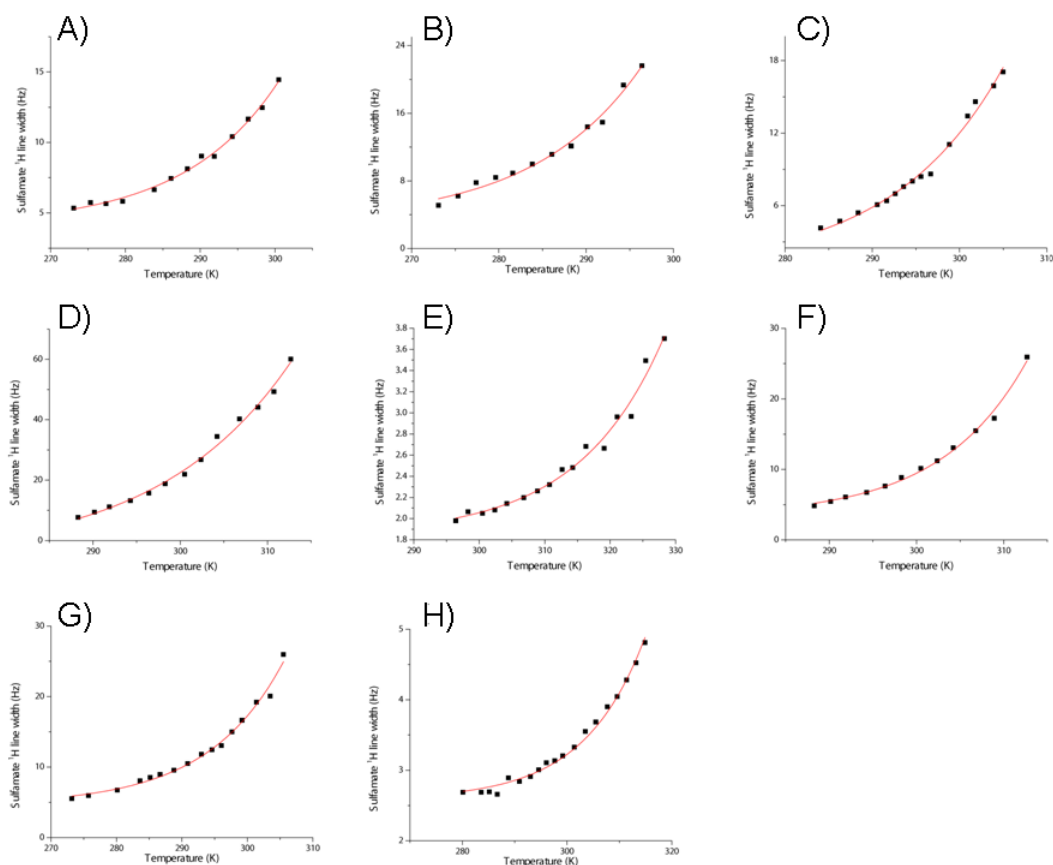


Figure 3.7. Plots used to determine the activation energy barrier, $\Delta G^\ddagger$, for the chemical exchange of the sulfamate protons with water for A) GlcNS(α), B) GlcNS(β), C) heparin disaccharide Δ UA(2S)-GlcNS(6S)-(α), D) Arixtra GlcNS(I) at pH 8.2, E) Arixtra GlcNS(III) at pH 8.2, F) Arixtra GlcNS(V) at pH 8.2, G) Arixtra GlcNS(I) at pH 7.4, H) Arixtra GlcNS(III) at pH 7.4. The Arixtra GlcNS(V) sulfamate proton exchanged too rapidly at pH 7.4 to acquire enough data points for a reliable exponential fit. Points correspond to the NH resonance line widths at half height determined by nonlinear least squares fitting of the experimental spectra with a Lorentzian line shape. The red line corresponds to the exponential fit of the data to the Eyring-Polanyi equation.

Table 3.2. Activation energies for the chemical exchange of the GlcNS sulfamate protons with water. Errors were calculated through a numerical estimate of the covariance matrix and correspond to ± 2.48 times the standard error for each parameter in this two parameter fit. To compensate for systematic errors, the calculated errors for each measurement were rounded up to ± 0.1 kcal/mol.

Saccharide	pH	$\Delta G^\ddagger$ (kcal/mol)
GlcNS(α)	7.4	15.6
GlcNS(β)	7.4	15.1
Disaccharide(α)	8.2	15.5
Arixtra GlcNS(I)	8.2	15.1
Arixtra GlcNS(III)	8.2	18.1
Arixtra GlcNS (V)	8.2	15.7
Arixtra GlcNS(I)	7.4	15.4
Arixtra GlcNS(III)	7.4	17.2

using NAMD 2.8 with the Glycam-06 force.^{33, 35, 36} Starting with Arixtra in the conformation it adopts when bound to Antithrombin-S195A factor Xa (PDB ID 2GD4),³² a 1 ns equilibration (300 K, 1.10325 bar) and 5 ns dynamics simulation (both with Glycam-06 forcefield and 1 fs timestep) were performed in a 12 Å water box consisting of 3493 water molecules (T1P3P forcefield) and ten sodium ions (ff99sb forcefield). Interestingly, during the molecular dynamics simulation transitions between both major ring conformations of the IdoA(2S) residue, 2S_0 and 1C_4 , were observed. Similar transitions for IdoA between the 1C_4 and 2S_0 conformations in molecular dynamics simulations of a heparin tetrasaccharide were observed over the course of 4 ns simulations that were either unrestrained or restrained using NMR residual dipolar couplings.⁵⁵ Previous solution NMR studies of Arixtra in aqueous solution⁵⁶ and bound to AT-III⁵⁷ have reported that the equilibrium between the IdoA(2S) ring conformations in the pentasaccharide favors the 2S_0 conformation over 1C_4 by a ratio of approximately 60:40. Figures 3.8A and 3.8B show representative structures selected from the IdoA(2S) 2S_0 and 1C_4 populations of conformers calculated over the 5 ns molecular dynamics simulation. The distances between the GlcNS(III) sulfamate hydrogen atom and the nearest 3-*O*-sulfo group oxygen atom are 2.22 Å and 2.46 Å, respectively, for the 2S_0 and 1C_4 conformations as shown in the figure.

Post-trajectory hydrogen bonding analysis was performed using a heavy atom cutoff distance of 3.5 Å and a hydrogen bond angle cutoff of 120°. A hydrogen bond

between the Arixtra GlcNS(III) NH group and the adjacent 3-*O*-sulfo group was observed in 89% of the calculated 2S_0 structures and 64% of the 1C_4 structures, with average N-O distances of 3.05 and 3.14 Å, respectively. The observation of a stronger and more persistent hydrogen bond in the 2S_0 conformer is significant. The 2-*O*-sulfo group of IdoA stabilizes the 2S_0 conformer of the bound pentasaccharide and enhances its contacts with the basic amino acids of the AT-III binding site.^{57, 58} A study of synthesized pentasaccharides in which the single L-iduronic acid was conformationally locked in either the 1C_4 , 4C_1 , or 2S_0 form showed that only the 2S_0 variant was able to fully activate antithrombin.⁵⁹ The results of our molecular dynamics simulation suggest that the GlcNS(III) NH - 3-*O*-sulfate hydrogen bond may contribute to the stabilization of the 2S_0 conformer of Arixtra in solution.

As shown in the two-dimensional Haworth structures presented in Figure 3.9, the molecular dynamics simulation also predicts stable hydrogen bonds between the Arixtra GlcNS(I) sulfamate group and the GlcA(II) residue C3 OH group for each conformer. Also for the 2S_0 conformer, a hydrogen bond was observed in 49% of structures between the GlcNS(III) sulfamate group and the C3 OH of IdoA(IV). In the 1C_4 conformer, an additional hydrogen bond was observed between the IdoA(IV) O5 and the GlcNS(V) C3 OH. Our measurements report only on the GlcNS(III) NH – 3-*O*-sulfo hydrogen bond, and future experiments focusing on the temperature dependence of the Arixtra hydroxyl resonances will be required to substantiate the additional OH hydrogen bonds suggested by the molecular dynamics simulation.

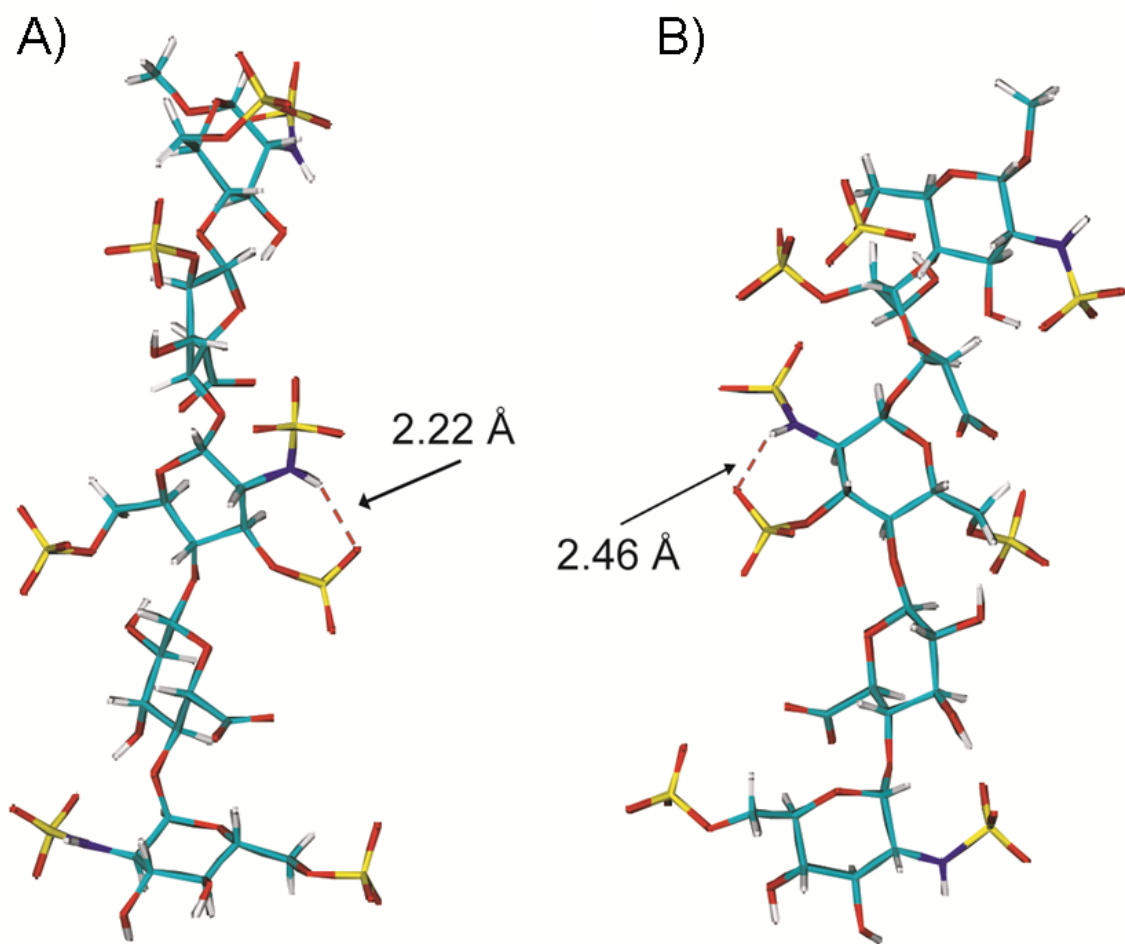


Figure 3.8. Representative structures from the 5 ns molecular dynamics simulation of the Arixtra pentasaccharide showing IdoA(2S) in the 2S_0 (A) and 1C_4 (B) conformations. The distances noted represent the average distance between the sulfamate protons and the oxygen of the sulfate at the 3-O position.

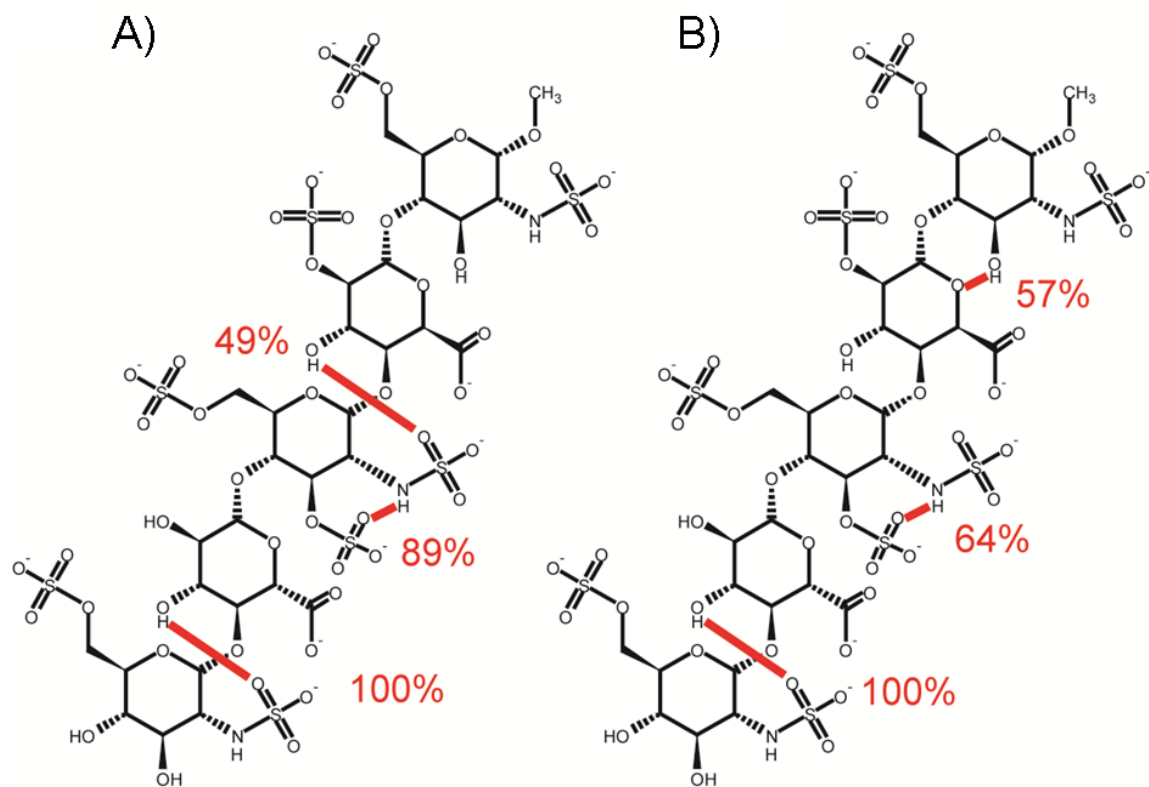


Figure 3.9. Representative structures from the 5 ns molecular dynamics simulation of the Arixtra pentasaccharide showing IdoA(2S) in the 2S_0 (A) and 1C_4 (B) conformations. The hydrogen bonds were calculated for each conformer using a heavy atom distance cutoff between the donor and the acceptor set of 3.5 Å and an angle cutoff of 120°. Percentage of structures in which the hydrogen bond is present is noted.

3.4 Conclusions

In this study the structural characteristics of the NH groups of GlcNS, Δ UA(2S)-GlcNS(6S), Δ UA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S), and the synthetic pentasaccharide Arixtra were probed through measurements related to the sulfamate proton exchange rate. Despite the biological significance of these compounds, we believe this to be the first study of the exchange properties of sulfamate protons of any GAG oligosaccharides. Clear and consistent differences in exchange rates of the sulfamate protons of the α and β anomers of the reducing end GlcNS residues were observed for the heparin mono-, di- and tetrasaccharide. The most significant outcome of this work is the substantial evidence for a persistent hydrogen bond between the sulfamate NH and the adjacent 3-*O*-sulfo group of the Arixtra GlcNS(3S)(6S) residue (GlcNS(III)). We believe this to be the first direct evidence for hydrogen bonding in a heparin-related saccharide in solution. A similar hydrogen bond is observed in the crystal structure of the AT-III bound Arixtra pentasaccharide,³² where there is a short heteroatom distance of 2.98 Å between the sulfamate nitrogen atom and the proximate 3-*O*-sulfo oxygen atom. Previous studies have noted the unusual conformation and “kink” in the AT-III binding heparin pentasaccharide at the GlcNS(III) residue.⁶⁰ The data presented herein suggest that this kink is due to the persistent hydrogen bond formed between the GlcNS(III) 3-*O* sulfo group and the adjacent sulfamate NH. The presence of this hydrogen bond, both in solution and in the crystal structure of the AT-III bound pentasaccharide, suggests that it is important in pre-organizing the pentasaccharide in a

conformation that favors binding, and is consistent with the well-established role of 3-O-sulfation in high-affinity binding.^{7, 8}

The results of this study further suggest that the sulfamate ¹H and ¹⁵N NMR resonances have untapped potential to provide important insights into biochemically important interactions in heparin and related GAGs. While this study focuses on using information obtained from the sulfamate resonances to evaluate factors of secondary structure of oligosaccharides, the [¹H, ¹⁵N] HSQC spectra of Arixtra in Figure 2.5B reveal that sulfamate ¹H and ¹⁵N chemical shifts are sensitive to local elements of primary structure. In Chapter 4 the ability of the sulfamate ¹H and ¹⁵N resonances to report on primary and secondary structure is further developed and used in the characterization of complex unfractionated and low molecular weight heparin and HS samples.

3.5 References

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CHAPTER FOUR

Characterizing the microstructure of heparin and heparan sulfate using *N*-sulfo-glucosamine ^1H and ^{15}N NMR chemical shift analysis

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Abstract: Heparin and heparan sulfate (HS) are members of a biologically important group of highly anionic linear polysaccharides called glycosaminoglycans (GAGs). Because of their structural complexity, the molecular-level characterization of heparin and HS continues to be a challenge. The work presented herein describes an emerging approach for the analysis of unfractionated and low molecular weight heparins as well as porcine and human-derived HS. This approach utilizes the previously untapped potential

of ^{15}N NMR to characterize these preparations through detection of the NH resonances of *N*-sulfoglucosamine residues. The sulfamate group ^1H and ^{15}N chemical shifts of six GAG microenvironments were assigned based on the critical comparison of selectively modified heparin derivatives, NMR measurements for a library of heparin-derived oligosaccharide standards, and an in-depth NMR analysis of the low molecular weight heparin enoxaparin through systematic investigation of the chemical exchange properties of NH resonances and residue-specific assignments using the $[^1\text{H}, ^{15}\text{N}]$ HSQC-TOCSY experiment. The sulfamate microenvironments characterized in this study include GlcNS(6S)-UA(2S), Δ UA(2S)-GlcNS(6S), GlcNS(3S)(6S)-UA(2S), GlcNS-UA, GlcNS(6S)-red $_{\alpha}$, and 1,6-anhydro GlcNS.

4.1 Introduction

NMR has proven to be an invaluable method for characterizing GAGs such as heparin, HS, chondroitin sulfate and hyaluronan as well as for the analysis of unfractionated heparin (UFH) and low molecular weight heparin (LMWH) pharmaceuticals.¹⁻³ $[^1\text{H}, ^{13}\text{C}]$ HSQC NMR spectra provide useful information about heparin structural features and substitution patterns and can be used to identify and quantify modifications such as those found in LMWH.⁴ While $[^1\text{H}, ^{13}\text{C}]$ HSQC spectra are routinely used for the characterization and quality assurance of pharmaceutical heparin samples, ^{15}N NMR has been underutilized for GAG characterization. In Chapters 2 and 3, we reported solution conditions facilitating detection of the prevalent heparin sulfamate groups and enabling measurement of ^1H and ^{15}N chemical shifts using the $[^1\text{H}, ^{15}\text{N}]$

HSQC experiment.⁵ We also demonstrated the use of the exchange rates of the sulfamate NH protons to reflect elements of secondary structure in a set of structurally defined heparin-derived oligosaccharides. This study led to the characterization of the first reported intramolecular hydrogen bond involving the 3-*O*- and 6-*O*-sulfated *N*-sulfoglucosamine sulfamate group proton and the adjacent 3-*O*-sulfate group in the pentasaccharide Arixtra.⁶

The work described herein extends the results of Chapters 2 and 3 to use ^1H and ^{15}N chemical shift analysis for the characterization of UFH and LMWH samples and HS biopolymers.⁵⁻⁷ This study was initiated by the systematic measurement of ^1H and ^{15}N chemical shifts of *N*-sulfoglucosamine (GlcNS) sulfamate groups in a variety of microenvironments using a library of oligosaccharide standards isolated from the LMWH enoxaparin and UFH enzymatic digests. Comparison of [^1H , ^{15}N] HSQC spectra of enzymatically and chemically modified heparins provided further evidence for residue-specific assignments. Measurement of [^1H , ^{15}N] HSQC spectra of enoxaparin as a function of pH probed the chemical exchange characteristics of the sulfamate protons in larger oligosaccharides, reinforcing initial assignments. Finally, the [^1H , ^{15}N] HSQC-TOCSY spectrum of enoxaparin was used to assign specific NH correlations through TOCSY cross peaks to sugar ring protons having chemical shifts characteristic of specific GlcNS microenvironments.

Enoxaparin was chosen for this study as it reflects a more diverse mixture of oligosaccharides than other LMWHs produced through β -elimination, such as tinzaparin, and does not suffer the loss of sulfamate groups in the formation of 2,5 anhydromannitol residues as in the reductive deamination process used to manufacture dalteparin.

4.2 Methods and Materials

4.2.1. Materials and reagents. HPLC-grade water used for NMR sample preparation was from Burdick and Jackson (Morristown, NJ). Deuterium oxide (99%), L-glutamine (^{15}N amide 98%), and ethylenediaminetetraacetic- d_6 (EDTA) were purchased from Cambridge Isotope Laboratories (Andover, MA). Dibasic sodium phosphate, sodium hydroxide, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, PA). DSS (2,2-dimethyl-2-silapentane-5-sulfonate- d_6 sodium salt) was purchased from Isotech (Miamisburg, OH). Calcium acetate, Tris-HCl, magnesium chloride, the trisulfated heparin-derived disaccharide (IS), porcine intestinal mucosa heparin and benzonase were purchased from Sigma-Aldrich (St. Louis, MO). Enoxaparin was obtained from the U.S. Pharmacopeia (Rockville, MD) while porcine heparan sulfate was purchased from Celsus labs (Cincinnati, OH). Bovine lung heparin was obtained from Upjohn Co. (Kalamazoo, MI) and de-2-O-sulfated porcine mucosal heparin was prepared as previously reported.⁸ The enzymes heparinase-I and heparinase-III were purchased from IBEX (Montreal, Quebec). Arixtra was provided by the University Pharmacy and Department of Pharmacy Administration of Semmelweis University. DMEM media without L-glutamine and fetal bovine serum were purchased from

Mediatech (Manassas, VA). Pronase was obtained from Roche (San Francisco, CA) and Triton X-100 was purchased from Alpha Aesar (Ward Hill, MA).

4.2.2. Heparinase-I digestion. A 1 g sample of porcine mucosal heparin was dissolved in 50 mL of 100 mM Tris buffer pH 6.8 with 2.5 mM calcium acetate. An aliquot of 0.5 IU of heparinase-I was added to the warmed solution and the reaction allowed to progress for 66 h at 28 °C. The reaction was quenched by placing the solution in boiling water for 5 min.

4.2.3. Heparinase-III digestion. A 1 g sample of porcine mucosal heparin was dissolved in 50 mL of 20 mM Tris buffer pH 7.5 with 50 mM NaCl. An aliquot of 0.5 IU of heparinase-III was added to the warmed solution and allowed to react overnight at 35 °C. This reaction was quenched by placing the solution in boiling water for 5 min.

4.2.4. Isolation of oligosaccharide standards. Oligosaccharides containing the 1,6-anhydro structure at the reducing end were isolated from enoxaparin, while the remainder were isolated from the heparinase-I digestion of porcine mucosal heparin described above. These samples were subjected to size fractionation by size-exclusion chromatography (SEC) followed by charge fractionation using strong anion chromatography (SAX). SEC separations were performed as previously described using a 3.0 x 200 cm column packed with Bio-Rad P-10 superfine gel (Bio-Rad Laboratories Hercules, CA) at 0.08 mL/min.¹ Fractions of 4 mL were collected and the UV absorbance measured at 232 nm.

The size-uniform oligosaccharide fractions isolated by SEC were subjected to SAX as previously described in Chapter 1 using a Dionex 500 ion chromatograph

equipped with a CarboPac PA1 column (9 mm x 25 cm) purchased from Dionex (Sunnyvale, CA).¹ The separation was carried out using two mobile phase buffers at a constant flow rate of 2.5 mL/min. Solution A consisted of 50 mM sodium phosphate pH 7.0 and solution B contained the same buffer as in A with 2.0 M NaCl. The gradient profile used was: 0 min: 100% A, 0% B; 10 min: 71% A, 29% B; 80 min: 39% A, 61% B, ending at 90 min. Individual SAX peaks were collected, pooled and desalted using a 1.6 cm x 70 cm Sephadex G10 superfine column (GE Healthcare, Pittsburgh, PA).

Oligosaccharides were identified as previously described based on ¹H NMR chemical shift assignments obtained from one-dimensional spectra, as well as the COSY, TOCSY, and ROESY spectra acquired in D₂O using a Bruker 600 MHz Avance spectrometer equipped with a BBI probe.^{1, 9} The TOCSY spectrum was used to identify each saccharide through chemical shift measurements for the protons within each sugar ring. The DQF-COSY spectrum confirmed scalar connectivities while the ROESY spectrum revealed the oligosaccharide sequence through detection of through-space connectivities across glycosidic linkages.

In addition to NMR, reversed-phase ion-pairing ultra performance liquid chromatography (UPLC) - mass spectrometry (MS) was used to confirm the oligosaccharide sequence. Isolated oligosaccharides were analyzed using a Waters Acquity UPLC with a Q-TOF MS detector in negative mode.^{10, 11} The separations were performed at 40 °C using a 2.1 mm x 100 mm Waters BEH C18 column containing 1.7 μm particles. The mobile phase buffers consisted of 10 mM acetic acid with 10 mM dibutylamine (DBA) as the ion-pairing reagent. Solution A was composed of 90%

H₂O/10% methanol and solution B was composed of 100% methanol. A linear elution gradient was used starting at 30% B and going to 55% B over 15 min. An equilibration time of 7 min was used between injections.

4.2.5. Isolation of HS from cell culture. HEK293T cells were grown to near confluence in 150 cm² vented flasks using 25 mL of DMEM without L-glutamine, 10% FBS and 3 mM of ¹⁵N-labelled L-glutamine (¹⁵N amide, 98%).²⁹ No antibacterial or antifungal supplements were used. Cells were lysed directly on the culture flask surface using 100 mM NaOH with 1% Triton X-100. The cell lysate and media were collected, combined and adjusted to neutral pH with concentrated HCl. CaCl₂ was added to make a final concentration of 2 mM and the sample treated with a final concentration of 0.5 mg/mL pronase and digested twice overnight at 50 °C. Following pronase digestion, the sample was adjusted to contain 2 mM MgCl₂ and incubated with benzonase at a final concentration of 2.5 mU/mL for 4 h at 37 °C. After enzymatic treatment the sample was twice passed through a 3 mL column of DEAE-Sephacel anion-exchange resin. The column was washed with 50 mL of wash solution (50 mM sodium acetate, 200 mM NaCl and 0.5% Triton X-100 at pH 6.0) and eluted with 6 mL of the wash solution containing 2 M NaCl. The eluent was treated with a solution containing 10% NaBH₄ in 2M NaOH at a ratio of 0.33 mL per 3 mL of column eluent and incubated overnight at -20 °C. The NaBH₄ was destroyed with enough acetic acid to neutralize the solution and the GAGs precipitated with 4 volumes of denatured ethanol. The solution was centrifuged at 9000 g and the pellet collected. The pellet was hydrated in a minimum volume of water and

dialyzed against HPLC-grade water using 3000 Da MWCO dialysis tubing from Spectrum labs (Rancho Dominguez, CA).

4.2.6. NMR parameters. The [^1H , ^{15}N] HSQC spectra (Figure 4.1) of enoxaparin, porcine mucosal heparin, bovine lung heparin, Celcus porcine HS and ^{15}N -enriched HEK293T-derived HS, and the spectrum of heparinase-III digested porcine mucosal heparin in Figure 4.3B were prepared by dissolving 75 mg of material in 600 μL of a solution containing 90% H_2O /10% D_2O with 10 mM sodium phosphate buffer at pH 8.2, 2 mM EDTA- d_6 and 2 mM DSS. Spectra were referenced to DSS per the IUPAC NMR referencing recommendation.¹² It is important to note that the sulfamate group NH resonance chemical shifts and line widths are temperature dependent, as discussed in Chapter 3,⁶ and for the purpose of consistency all [^1H , ^{15}N] HSQC spectra in this work were acquired at 20 $^\circ\text{C}$ unless otherwise noted. Samples were placed into a 5 mm NMR tube and [^1H , ^{15}N] HSQC spectra measured using a Bruker 600 Avance spectrometer equipped with a BBI probe. These spectra were acquired in 4096 complex points in t_2 with 128 scans coadded for each of the 160 t_1 increments. The spectral window was 9 ppm in F2 centered on the HOD signal and 15 ppm in the F1 centered at 92 ppm. A $^1J_{(\text{NH})}$ of 80 Hz was used with a 2.0 s relaxation delay. The spectra were linear predicted to 224 points in F1, zero-filled to 4096 x 512 points and apodized using the $\cos^2$ window function in both dimensions. Spectra were typically acquired as overnight experiments with 15 hours being a representative measurement time. Spectra were measured at 20 $^\circ\text{C}$ to minimize peak broadening due to chemical exchange and prevent the slight variation in chemical shifts that could result from temperature fluctuations. In the future, it may

prove useful to acquire GAG [^1H , ^{15}N] HSQC spectra at even lower temperatures or using higher field cryoprobe-equipped spectrometers to facilitate detection of less abundant sulfamate group microenvironments. The [^1H , ^{15}N] HSQC spectra of de-2-*O*-sulfated heparin was acquired using a sample mass of 17 mg in 325 μL of phosphate buffer, as described above, in a 5 mm D_2O matched Shigemi tube (Shigemi Inc, Allison Park, PA). The acquisition parameters were identical except that 176 t_1 increments were used. The [^1H , ^{15}N] HSQC spectra of bovine lung heparin was acquired in a 3 mm NMR tube using a cryoprobe-equipped 500 MHz Bruker Avance spectrometer at the G. Ronzoni Institute in Milan Italy.

To maximize the measurement sensitivity, [^1H , ^{15}N] HSQC spectra of the oligosaccharide standards were acquired using a 1.7 mm capillary insert with a sample volume of 35 μL centered in a 3 mm NMR tube (New Era Enterprises, Sevierville TN) using a Teflon holder. Sample buffer conditions mimicked those described above except that the sample was prepared in 100% H_2O with the spectrometer lock provided by 100% D_2O in the 3 mm tube's annular volume. Depending on the availability of the isolated oligosaccharides, samples were prepared using masses ranging from 0.2 mg to 2 mg dissolved in 35 μL . Oligosaccharide [^1H , ^{15}N] HSQC spectra were measured using the aforementioned 600 MHz Bruker spectrometer for samples of 0.4 mg or more; compounds isolated in quantities of 0.2 mg or less were measured at the Complex Carbohydrate Research Center (CCRC) of the University of Georgia using a 600 MHz Varian spectrometer equipped with a 3 mm coldprobe. NMR parameters were similar to

those describe above, with t_1 increments ranging from 128 to 320 and numbers of scans ranging 176 to 320 depending on the available mass.

The [^1H , ^{15}N] HSQC-TOCSY spectrum was measured using 200 mg of enoxaparin in 350 μL of 90% H_2O /10% D_2O buffer in a D_2O matched Shigemi tube (Shigemi Inc, Allison Park, PA). The spectrum was acquired in 4096 complex points in t_2 with 256 scans coadded for each of 148 t_1 increments. A spectral window of 7 ppm in F2 and 13 ppm in F1 were used with a relaxation delay of 2.0 s. A $^1J_{(\text{NH})}$ value of 80 Hz was used with a TOCSY mixing time of 100 ms. The spectrum was zero filled to 8192 x 512 points and apodized using $\cos^2$ in both dimensions.

4.3. Results and Discussion

4.3.1. Defining GlcNS microstructures in heparin and HS. In Chapter 2 the sensitivity of the ^1H and ^{15}N NMR GlcNS sulfamate group chemical shifts to structure was evaluated for the pentasaccharide Arixtra suggesting their potential for GAG structural characterization. In this chapter we extend this chemical shift analysis to the structurally more complex samples of UFH, LMWH and HS. The 600 MHz [^1H , ^{15}N] HSQC spectra of enoxaparin, UFH from porcine intestinal mucosa, UFH from bovine lung, porcine HS, and HS derived from ^{15}N enrichment of cultured HEK293T cells are presented in Figure 4.1. The peaks observed in these spectra are designated using Roman numerals with the corresponding chemical shifts presented in Table 4.1. Figure 4.1 highlights the similarities and differences in the spectra measured for GAGs obtained from the various sources. The porcine mucosal heparin, bovine lung heparin and

enoxaparin spectra all have peaks designated as I. In the enoxaparin spectrum, however, this correlation appears to be split into two distinct populations, with the peak having a ^{15}N chemical shift of 94.6 ppm labeled as II. Cross peak III is particularly sharp and is present in enoxaparin as well as both intact heparin samples, but is not detected in the HS spectra. Peak IV is consistent throughout all five spectra in Figure 4.1 and is present in both heparin and HS samples. The LMWH enoxaparin also has a unique correlation designated as V. At higher levels of vertical scale than used in Figure 4.1, a weak peak corresponding to V can also be observed in the porcine intestinal mucosa and bovine lung heparin spectra, but the signal is of low intensity and close to the noise floor. Additional unlabeled peaks were observed in the $[^1\text{H}, ^{15}\text{N}]$ HSQC spectrum of enoxaparin (Figure 4.1A). These peaks were not identified in this work and will require further investigation to allow their assignment. It is also likely that additional, lower abundance peaks, are present below the noise floor of the spectra shown in Figure 4.1 and the use of a more sensitive spectrometer would permit their detection.

4.3.2 Chemical shift analysis of GlcNS containing oligosaccharide standards. To better understand the structural characteristics or microenvironments that give rise to the observed correlations in the $[^1\text{H}, ^{15}\text{N}]$ HSQC spectra of the intact GAG polymers, a series of oligosaccharide standards were isolated by SEC and SAX from porcine mucosal heparin digested with heparinase-I and enoxaparin and characterized by ^1H NMR and UPLC-MS with the invaluable assistance of Consuelo N. Beecher. The $[^1\text{H}, ^{15}\text{N}]$ HSQC spectrum of each standard was measured and the chemical shifts of the isolated di, tetra and hexasaccharides along with the Arixtra pentasaccharide, which is used clinically, are

summarized in Table 4.2 with their structures illustrated in Figure 4.2. Several preliminary peak assignments in the GAG [^1H , ^{15}N] HSQC spectra shown in Figure 4.1 can be made by comparison to the results in Table 4.2, as described below.

4.3.3 Chemical shifts of the terminal GlcNS residues. The α -anomer of the *N*-sulfoglucosamine residue of the reducing end of the GAG chains is the easiest peak to assign using the information in Table 4.2. The sulfamate proton resonance of the β -anomeric form of the reducing end GlcNS was not detected in any heparin or HS preparation, likely due to its lower abundance and the greater line widths observed in the spectra of the oligosaccharide standards. The ^1H and ^{15}N chemical shifts of the disaccharide α -anomer along with those of the reducing ends of the isolated tetrasaccharides are consistent with the corresponding peak V in the enoxaparin HSQC spectrum. This peak is much less intense in spectra measured for preparations of the higher molecular weight UFH and is not observed in the HS samples, which contain a proportionally smaller fraction of reducing ends. As a result of the chemical depolymerization steps used in its preparation, the LMWH heparin enoxaparin has a lower average molecular weight resulting in more reducing ends per unit mass.

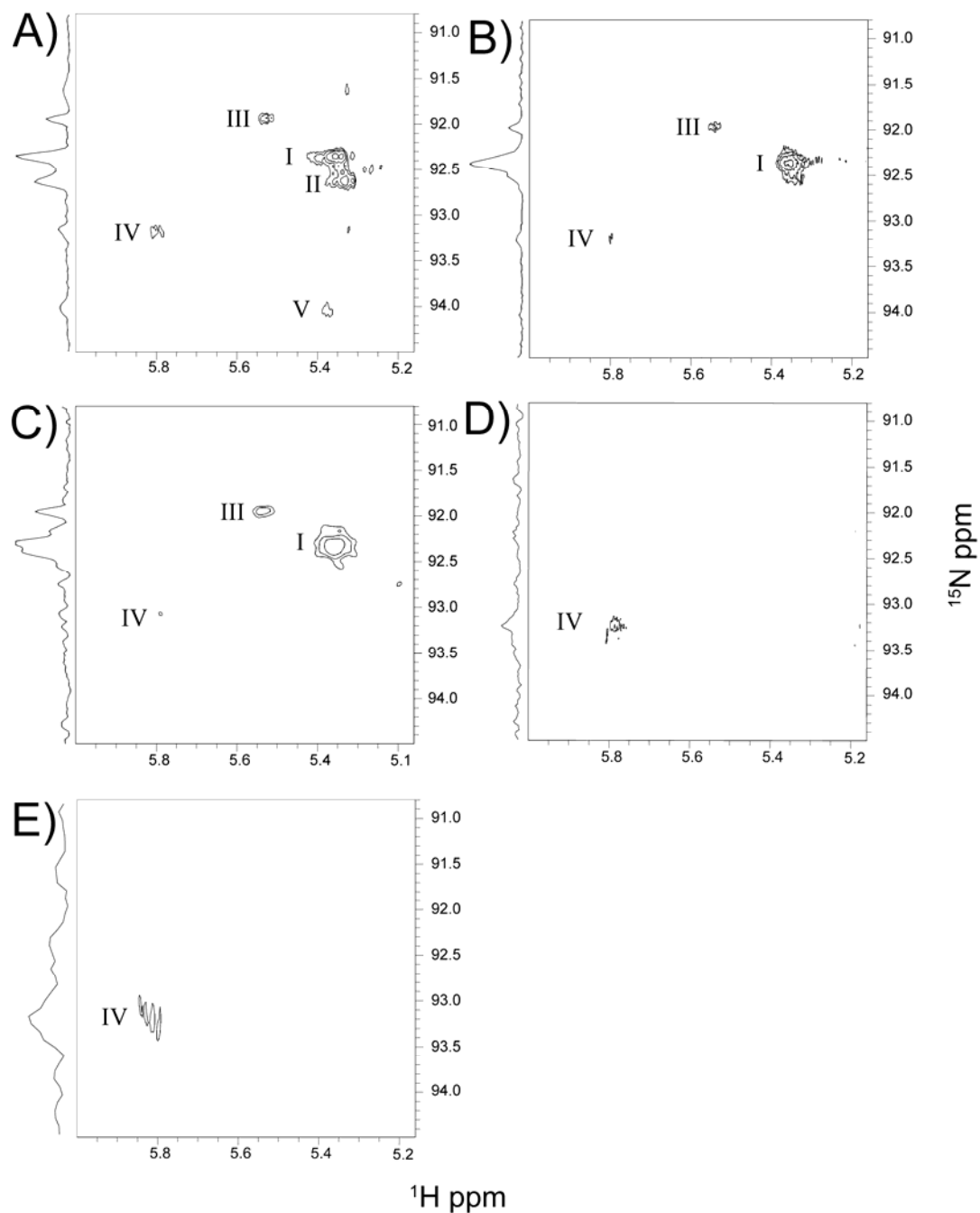


Figure 4.1. [^1H , ^{15}N] HSQC spectra of GAG samples: a) enoxaparin, b) porcine intestinal mucosa heparin, c) bovine lung heparin, d) Celsus porcine HS, and e) ^{15}N -enriched HEK293T-derived HS. Spectra were measured at 20° C in 90% $\text{H}_2\text{O}/10\%$ D_2O .

Table 4.1. Chemical shifts determined from the 600 MHz [^1H , ^{15}N] HSQC spectra shown in Figure 4.1.

Sample	Designation	^1H (ppm)	^{15}N (ppm)
Enoxaparin	I	5.35	92.4
	II	5.33	92.6
	III	5.53	92.0
	IV	5.80	93.2
	V	5.38	94.0
	VI	5.38	96.1
Porcine mucosal heparin	I	5.35	92.4
	III	5.54	92.0
	IV	5.80	93.2
Bovine lung heparin	I	5.39	91.9
	III	5.58	92.2
	IV	5.83	93.0
Commercial HS	IV	5.81	93.2
HEK293T-derived HS	IV	5.81	93.2

Table 4.2. Summary of peak assignments and ^1H and ^{15}N chemical shifts determined for a series of oligosaccharide standards. Residue number is counted from the non-reducing end. Spectra were acquired at 20 °C for all compounds except Hexa, which was measured at 17 °C.

I.D.	Oligosaccharide Structure	Residue	^1H (ppm)	^{15}N (ppm)
Di	$\Delta\text{UA}2\text{S-GlcNS}6\text{S}$	II- α	5.44	94.2
Tetra-1	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}6\text{S}$	II	5.39	92.6
		IV- α	5.34	94.1
Tetra-2	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}$	II	5.17	92.8
		IV- α	5.36	94.0
Tetra-3	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA-GlcNS}6\text{S}$	II	5.79	93.3
		IV- α	5.38	93.7
Tetra-4	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-GlcA}2\text{S-GlcNS}$	II	5.78	92.9
		IV- α	5.42	94.0
Tetra-5	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-GlcA-GlcNS}6\text{S}$	II	5.85	93.4
		IV- α	5.44	94.1
Tetra-6	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}(1,6\text{-anhydro})$	II	5.20	92.8
		IV	5.38	96.1
Tetra-7	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-ManNS}(1,6\text{-anhydro})$	II	5.21	92.8
		IV	5.75	95.9
Tetra-8	$\Delta\text{UA}2\text{S-GlcNS-IdoA}2\text{S-GlcNS}6\text{S}$	II	5.30	92.8
		IV	5.41	94.1
Arixtra	$\text{GlcNS}6\text{S-GlcA-GlcNS}3\text{S}6\text{S-IdoA}2\text{S-GlcNS}6\text{SOMe}$	I	5.88	92.8
		III	5.60	92.1
		V	5.41	93.9
Hexa	$\Delta\text{UA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}6\text{S-IdoA}2\text{S-GlcNS}6\text{S}$	II	5.27	92.7
		IV	5.32	92.4
		VI- α	5.37	94.2

The peak arising from the non-reducing end of enoxaparin oligosaccharides can be assigned by comparison with the hexasaccharide chemical shifts in Table 4.2. This hexasaccharide has two internal GlcNS(6S) residues and one residue at the reducing end. Sulfamate chemical shifts of the GlcNS(6S) residue adjacent to the non-reducing end 2-*O*-sulfo unsaturated uronic acid, Δ UA(2S), (5.27 and 92.7 ppm) and the internal residue linked to the unmodified IdoA(2S) (5.32 and 92.4 ppm) are strikingly similar to the peaks II and I, respectively, in the enoxaparin spectrum. Assigning peak II as the GlcNS(6S) residue directly adjacent to the Δ UA(2S) is supported by the chemical shifts measured for the hexasaccharide. Peak I is tentatively assigned as an internal GlcNS residue as it is the most intense cross peak in both UFH samples (Figure 4.1B and C) and closely matches the chemical shifts of the internal GlcNS(6S) within the hexasaccharide. These assignments are further supported by the fact that peak II is observed only in the enoxaparin spectrum (Figure 4.1A), the only heparin sample examined in which Δ UA residues, resulting from the depolymerization process, are expected at the non-reducing ends of the oligosaccharide chains.

4.3.4. Effect of uronic acid 2-*O*-sulfation on GlcNS chemical shifts. The chemical shifts in Table 4.2 can also provide information regarding the effect of 2-*O*-sulfation of the neighboring uronic acid by comparing tetrasaccharides differing only by a single factor such as saccharide identity (IdoA or GlcA) or a single substitution. Comparing the chemical shifts of the sulfamate group of the internal GlcNS(6S) of Tetra-3 (5.79 and 93.3 ppm) to that of Tetra-5 (5.85 and 93.4 ppm) reveals only subtle differences in the ^1H and ^{15}N chemical shifts of a GlcNS(6S) residue linked to the H4 of

an IdoA or a GlcA. This suggests that the sulfamate chemical shifts do not reflect the configuration of the neighboring uronic acid.

For some tetrasaccharides in Table 4.2, the sulfamate chemical shifts appear to reflect the influence of 2-*O*-sulfation of the adjacent uronic acid. For example, both the ^1H and ^{15}N chemical shifts of the internal GlcNS(6S) of Tetra-1 (5.39 and 92.6 ppm) and Tetra-3 (5.79 and 93.3 ppm) reflect whether the adjacent IdoA residue is 2-*O*-sulfated. A similar grouping is apparent in the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC spectra in Figure 4.1 suggesting that the peak I may be due to sulfamate groups adjacent to a 2-*O*-sulfated uronic acid while peak IV arises from sulfamate groups linked to uronic acid residues that are unmodified at the 2-*O* position. The chemical shifts of the corresponding sulfamate group in the GlcA containing oligosaccharide Tetra-5 (5.85 and 93.4 ppm) support this hypothesis, however the trend appears to break down for Tetra-2 (5.17 and 92.8 ppm) which lacks 6-*O*-sulfation of the reducing end GlcNS. Removal of the sterically hindering 6-*O* sulfate group may increase the freedom of motion of the reducing end saccharide impacting the chemical shifts of the internal sulfamate NH group.

Additional experiments were performed for two structurally modified heparins to probe potential end effects in the oligosaccharide data in Table 4.2 and to further test whether the sulfamate chemical shifts in larger saccharide chains systematically reflect 2-*O*-sulfation of the adjacent uronic acid. Figure 4.3A presents the $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ HSQC spectrum of unfractionated porcine intestinal mucosa heparin as a reference for comparison of the effects of these structural modifications. The spectrum in Figure 4.3B was measured for a porcine intestinal mucosa heparin sample digested using heparinase-

III, which is reported to specifically cleave the heparin chain between GlcNS or GlcNAc and GlcA or IdoA residues that lack sulfation at the 2-O position.¹³ Digestion with heparinase-III should primarily affect the chemical shifts of GlcNS residues bonded to unsulfated uronic acid residues, as these are the locations where cleavage will occur. Comparison of the [^1H , ^{15}N] HSQC spectrum of heparinase-III digested heparin (Figure 4.3B) with that of the intact polymer (Figure 4.3A) reveals that only peak IV appears to be affected by enzymatic cleavage, consistent with its assignment to GlcNS residues adjacent to an unsulfated uronic acid. To confirm this assignment, a [^1H , ^{15}N] HSQC spectrum (Figure 4.3C) was acquired for a heparin sample for which the uronic acid 2-O sulfate groups were chemically removed.¹⁴ This modification should primarily affect the sulfamate group chemical shifts of GlcNS residues bonded to a 2-O-sulfated uronic acid. Comparison of Figure 4.3C with the spectrum of unmodified heparin (Figure 4.3A) indicates that the only peak not affected by 2-O-desulfation is the peak designated IV, confirming its assignment as GlcNS-UA. The results in Figure 4.3 further suggest that the peaks I and III arise from internal GlcNS residues bonded to 2-O-sulfated uronic acid residues.

4.3.5 Effect of 3-O-sulfation on GlcNS chemical shifts. In the [^1H , ^{15}N] HSQC spectra in Figure 4.1 the ^1H and ^{15}N chemical shifts of peak III for enoxaparin (5.53 and 92.0 ppm), porcine mucosal heparin (5.54 and 92.0 ppm) and bovine lung heparin (5.58, 92.2 ppm) are similar to those of the GlcNS(3S)(6S) residue of Arixtra (Table 4.2, 5.60 and 92.0 ppm). This suggests that peak III corresponds to a 3-O-sulfated GlcNS residue and can be preliminarily assigned as GlcNS(3S)-UA(2S). The unique ^1H and ^{15}N

chemical shifts of 3-*O*-sulfated GlcNS are likely due to the proximity of the 3-*O*-sulfate group and its intraresidue hydrogen bond involving the sulfamate group as the proton donor, as described in Chapter 3.⁶ As a result of this stable hydrogen bond, the Arixtra GlcNS(3S)(6S) NH exchanges slowly with water therefore its resonance can be detected over a much wider pH range than the other sulfamate NH groups of Arixtra. This characteristic exchange profile can also be observed in the [¹H, ¹⁵N] HSQC spectra of enoxaparin (Figure 4.4) as peak III appears unchanged through the pH range 7.00 to 9.42. The assignment of the most rapidly exchanging sulfamate protons to the reducing end GlcNS residues (peak V) is also confirmed by the results in Figure 4.4 as this correlation is observed only in the spectra measured at pH 7.85 and 8.20 closely matching the exchange behavior of the reducing end GlcNS sulfamate group protons described in Chapter 3.

4.3.6. HSQC-TOCSY correlations confirm resonance assignments. To further support the assignments of the [¹H, ¹⁵N] HSQC spectra presented in Figure 4.1, two-dimensional [¹H, ¹⁵N] HSQC-TOCSY spectra were acquired (Figure 4.5).⁷ The HSQC-TOCSY experiment detects the protons within the GlcNS spin system and correlates them to the sulfamate group ¹⁵N chemical shifts, reporting on the structural characteristics of the GlcNS residues responsible for the various peaks in the [¹H, ¹⁵N] HSQC spectra.¹⁵

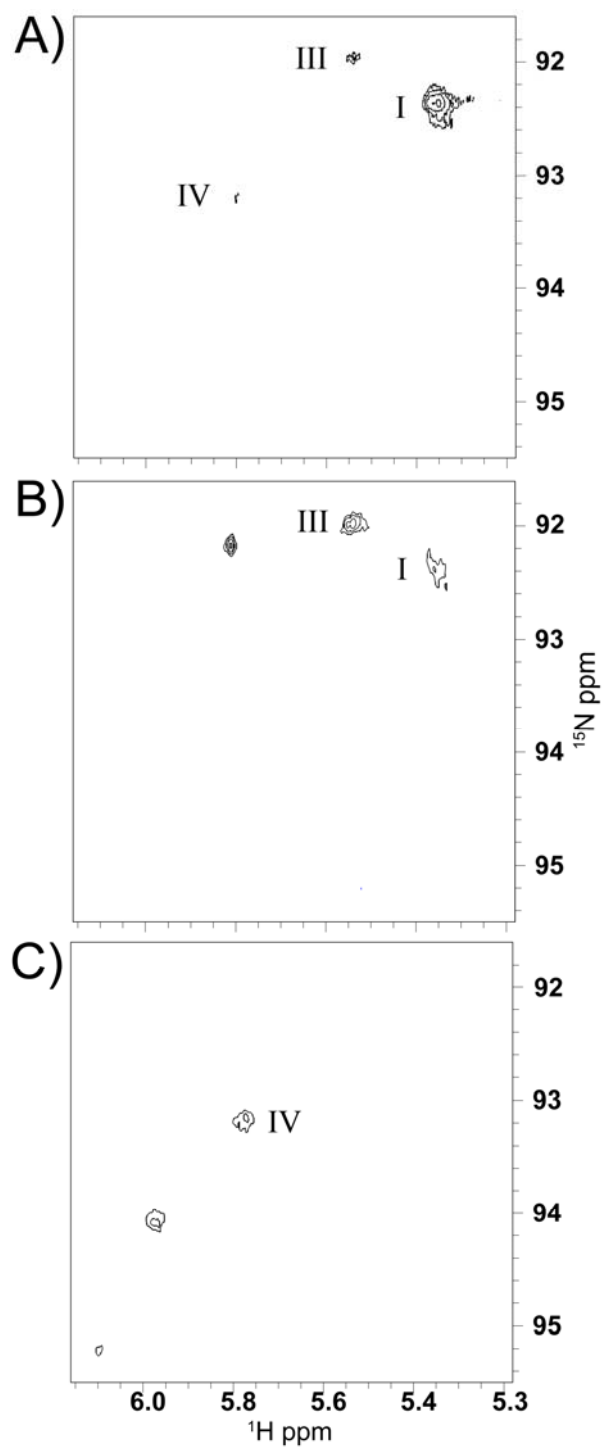


Figure 4.3. [^1H , ^{15}N] HSQC spectra of a) porcine intestinal mucosa heparin, b) porcine intestinal mucosa heparin treated with heparinase-III, c) 2-O-desulfated porcine intestinal mucosa heparin. All spectra were measured at 20° C.

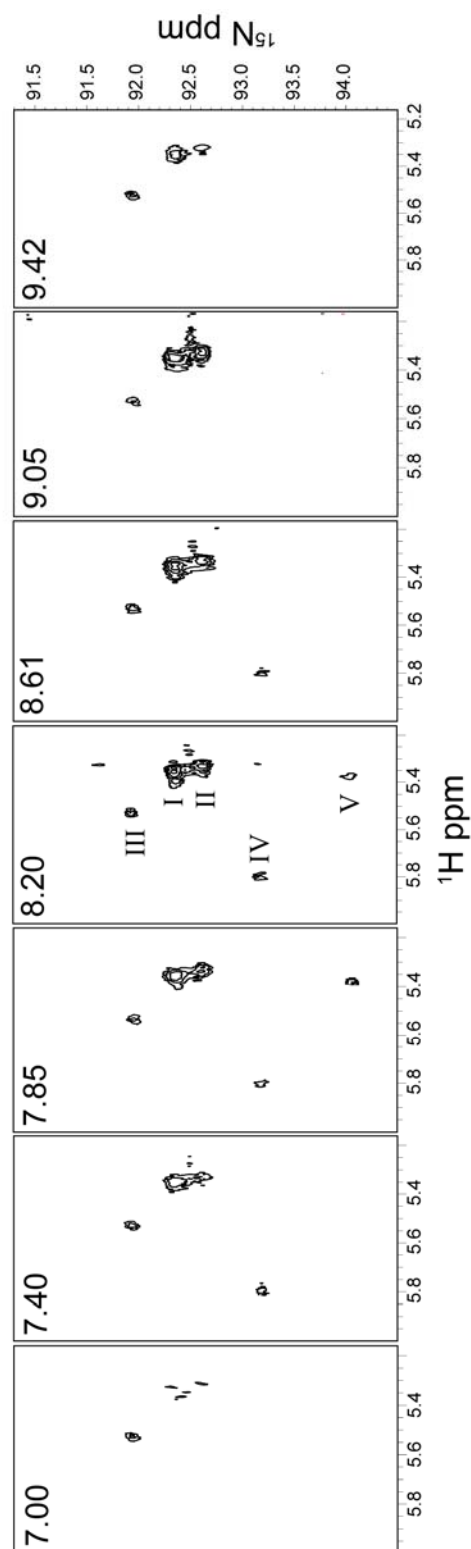


Figure 4.4. [^1H , ^{15}N] HSQC spectra of enoxaparin measured as a function of pH. Sample pH is noted in each spectrum. Spectra were measured at 20° C.

While this experiment suffers from somewhat lower sensitivity than the simpler [^1H , ^{15}N] HSQC experiment, this is not a limitation for enoxaparin as it is readily available and highly soluble.

The [^1H , ^{15}N] HSQC-TOCSY spectrum in Figure 4.5 shows all the sulfamate NH peaks observed in Figure 4.1, with only peak IV observed at too low of intensity to give rise to detectable TOCSY correlations. The [^1H , ^{15}N] HSQC-TOCSY spectrum confirms the assignment of peak II, which is only observed in enoxaparin (Figure 4.1A). The chemical shifts of the carbon-bound protons correlated to sulfamate peaks I and II are nearly identical with the exception of the H4 ring protons. The higher chemical shift of the H4 proton for sulfamate peak II supports its assignment to a GlcNS linked to a ΔUA residue.¹⁶ Another observation in Figure 4.5 of particular significance is the proton correlation at 4.39 ppm in F2 for NH peak III. In heparin this chemical shift is highly characteristic of the H3 proton of a 3-O-sulfated GlcNS^{7, 17-19} Therefore the HSQC-TOCSY spectrum provides additional spectroscopic evidence for the assignment of peak III as GlcNS(3S)-UA(2S), a required sequence for heparin's antithrombotic activity.

Likely due to the higher enoxaparin concentration used, the [^1H , ^{15}N] HSQC-TOCSY spectrum in Figure 4.5 exhibits an additional sulfamate peak that was not observed in the HSQC spectrum in Figure 4.1A. Labeled as peak VI, this correlation has a ^{15}N chemical shift of 96.1 ppm. Peak VI is correlated with two proton resonances at 5.38 and 5.60 ppm in Figure 4.5. Comparing the chemical shifts for peak VI to the values in Table 4.2, the NH correlation at 5.38 and 96.1 ppm can be assigned to a reducing end 1,6-anhydro residue. The additional ^1H cross peak at 5.60 ppm is also consistent with the

value measured for the H1 (5.602 ppm) of the 1,6-anhydro GlcNS terminated Tetra-6. Detection of this unique microenvironment and its assignment to a 1,6-anhydro GlcNS residue showcases the utility of the HSQC-TOCSY experiment as the anhydro structures of enoxaparin can include 1,6-anhydro glucosamine as well as 1,6-anhydro mannosamine. According to Mascellani et al., typically only 15-25% of the chains contain an anhydro structure at the reducing end corresponding to only about 1.4-2.3% of the total mass of the LMWH.²⁰

4.3.7. Effect of 6-*O*-sulfation on GlcNS chemical shifts. Limtiaco et al. reported that sulfamate group ¹H and ¹⁵N chemical shifts are relatively insensitive to 6-*O*-sulfation in GlcNS monosaccharides. The results presented herein, however, provide evidence that sulfamate chemical shifts in larger oligosaccharides, such as those found in enoxaparin, may be sensitive to glucosamine 6-*O*-sulfation. While both the H5 and H6 protons can be used to report on substitution at the 6-*O* position, the H5 protons are fewer bonds away from the sulfamate group and thus give stronger signals in the [¹H, ¹⁵N] HSQC-TOCSY spectrum. The [¹H, ¹⁵N] HSQC-TOCSY spectrum in Figure 4.5 shows correlations from each of the sulfamate peaks I, II, III, and V to carbon-bound sugar resonances at 4.01 ppm, 4.02 ppm, 4.12 ppm, and 4.14 ppm respectively. The sulfamate correlations for

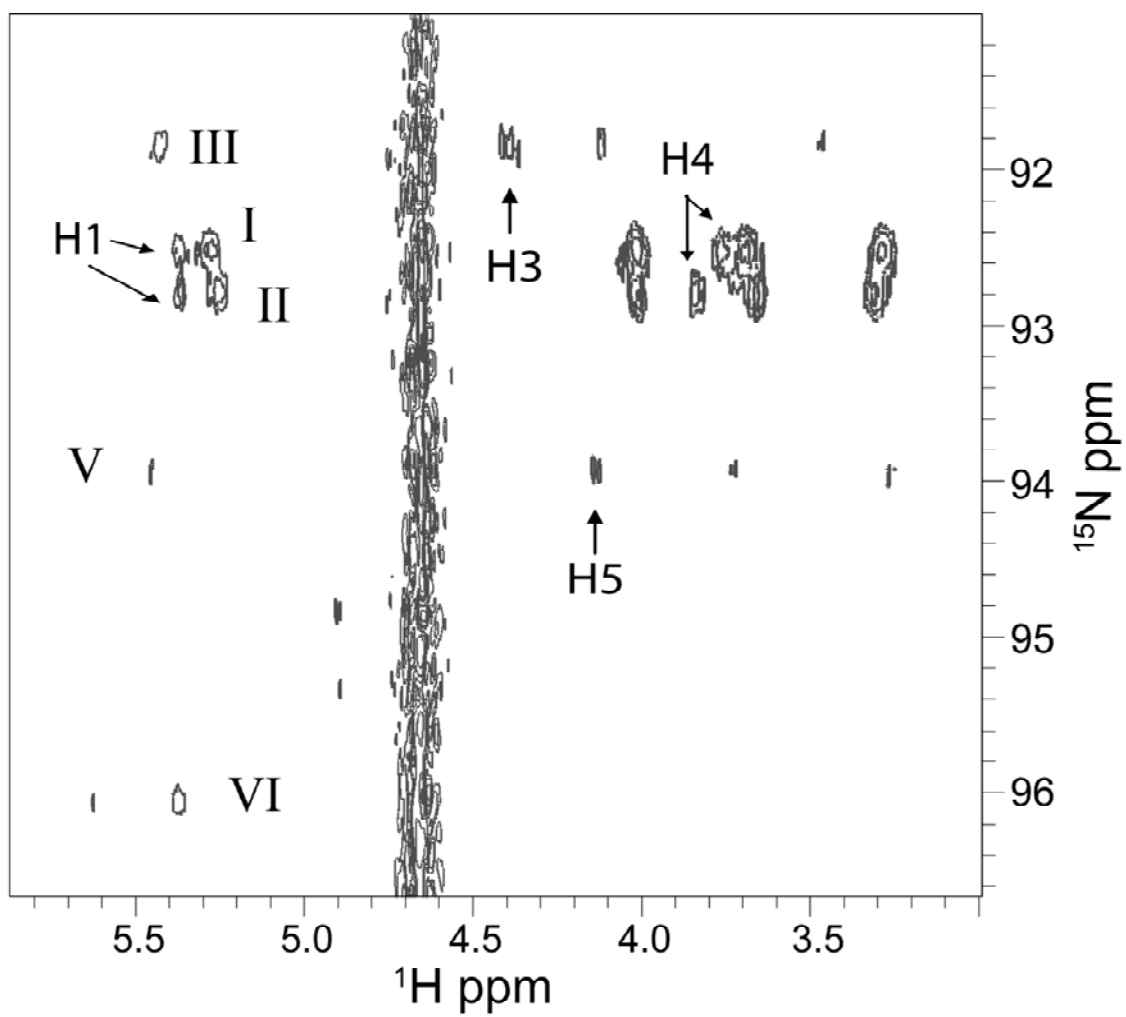


Figure 4.5. 2D [^1H , ^{15}N] HSQC-TOCSY spectrum of enoxaparin measured at pH 8.2 at 20° C. Sulfamate NH correlations are labeled with Roman numerals.

peaks I and II at 4.01 ppm and 4.02 ppm are consistent with published values for the H5 proton of GlcNS(6S). The resonance correlated to peak III also closely matches the H5 chemical shift of the GlcNS(3S)(6S) found in Arixtra⁷ and the resonance at 4.14 ppm is characteristic of the H5 proton of a GlcNS(6S) at the reducing end¹ which is consistent with the assignment of sulfamate peak V as the reducing end GlcNS.

Most glucosamine residues in heparin are sulfated at both the 6-*O* and *N* positions, and GlcNS residues lacking 6-*O* sulfation are expected to be relatively minor constituents of LMWH and UFH samples. To determine the impact of 6-*O* sulfation on GlcNS ¹H and ¹⁵N NMR chemical shifts, the HSQC spectrum was measured for Tetra-8, a relatively minor component of the tetrasaccharide SEC fraction used for isolation of oligosaccharide standards. As shown in Table 4.2, the Tetra-1 and Tetra-8 oligosaccharides differ in their structures only by substitution at the 6-*O* position of the internal GlcNS residue. For Tetra-1 for which the internal GlcNS is 6-*O* sulfated, the ¹H and ¹⁵N chemical shifts are 5.39 ppm and 92.6 ppm, respectively, while for Tetra-8 these values are 5.30 ppm and 92.8 ppm. This result demonstrates that the sulfamate chemical shifts of internal GlcNS residues are indeed sensitive to 6-*O* sulfation. Table 4.3 summarizes the ¹H and ¹⁵N resonance assignments for the heparin and HS samples investigated.

4.4. Conclusions.

This work provides the first characterization data for the ^1H and ^{15}N resonances of the GlcNS sulfamate groups of unfractionated and low molecular weight heparins, and heparan sulfate. Although measurements of isolated oligosaccharide standards were critical to the success of this study, the spectra of heparins modified structurally by 2-O-desulfation and heparinase-III digestion provided key insights leading to definitive resonance assignments. By assigning the identities of the microstructures responsible for the observed ^1H and ^{15}N correlations, these results pave the way for the analytical characterization of heparin and HS in terms of specific structural traits. This approach is intended to complement the use of [^1H , ^{13}C] HSQC NMR for GAG analysis and characterization. Low molecular weight heparin [^1H , ^{15}N] HSQC spectra are significantly less complicated than their ^{13}C analogs, while still reporting on the major structural components of the sample.

The [^1H , ^{15}N] HSQC-TOCSY spectrum of enoxaparin was invaluable in completing and confirming the resonance assignments of all the GAGs studied. The HSQC-TOCSY experiment shows considerable promise for quality assurance applications, including detection of unusual or unexpected structures. Although this experiment is somewhat less sensitive than the standard gradient HSQC pulse sequence, the sensitivity of these measurements could be improved through the use of a cryogenically-cooled probe or magnetic field higher than the 600 MHz instrument used in this study.

Table 4.3 Summary of sulfamate peak assignments for the [^1H , ^{15}N] HSQC spectra presented in Figure 4.1.

Sulfamate peak	Microenvironment
I	GlcNS(6S)-UA(2S)
II	Δ UA(2S)-GlcNS(6S)
III	GlcNS(3S)(6S)-UA(2S)
IV	GlcNS-UA
V	GlcNS(6S)-red $_{\alpha}$
VI	1,6-anhydro GlcNS

Relative quantitation of GlcNS(3S)(6S) [^1H , ^{15}N] HSQC correlations could perhaps be used to gauge potency of heparin products as anticoagulants and comparison of intensities of peaks I and IV could provide information about the overall extent of 2-*O*-sulfation in a sample. It may also be possible to quantify the extent of heparin depolymerization based on the relative intensities of sulfamate [^1H , ^{15}N] HSQC peaks at the reducing and non-reducing ends of the biopolymers. Additionally, depolymerization modifications like the 1,6-anhydro structures could be readily monitored in LMWH samples using [^1H , ^{15}N] HSQC experiments.

One challenge to using [^1H , ^{15}N] HSQC spectra for quantitative determination of different GlcNS microstructures in complex heparin samples is the possibility that different local environments may give rise to differences in sulfamate group solvent exchange rates. Further study is required to evaluate the extent to which NH exchange rates of structurally different GlcNS residues vary under measurement conditions selected to minimize solvent exchange, and the impact of these differences on quantitative determinations.

The work presented in this chapter builds on the results discussed in Chapters 2 and 3 and would not have been possible without the development of this prior knowledgebase. By determining solution conditions that facilitate the detection of the sulfamate NH resonances, we developed a new tool for characterizing GlcNS containing oligo- and polysaccharides using their ^1H and ^{15}N chemical shifts. The NMR line shape analysis of the sulfamate ^1H resonances described in Chapter 3 allowed us to use the pH dependence of NH-solvent exchange to verify the assignments for the slowly exchanging

GlcNS(3S)(6S) and the solvent exposed reducing end GlcNS(6S) residue. The [^1H , ^{15}N] HSQC-TOCSY NMR spectra allowed us to link the sulfamate chemical shifts to other carbon-bound protons and use these resonances to determine the location of critical O-sulfo substitutions. This work contributes a novel characterization scheme for heparin, a complex polymeric pharmaceutical drug, whose structure and activity been long studied since the discovery of its anticoagulative properties in 1916 by Jay McLean at John's Hopkins University.²¹

The analytical method outlined in this chapter provides information on the microstructures found in UFH, HS and LMWH independent of oligosaccharide size or molecular weight distribution. When the analytical goal is aimed at characterizing GAG mixtures in terms of molecular weight distribution, constituent oligosaccharides or for profiling LMWH preparations, it may be necessary to perform chromatographic separations of the mixtures to tease out the identity and relative abundance of individual oligosaccharides. In Chapter 5 we aim to adapt and refine separation methods capable of providing orthogonal information for the characterization of LMWH samples at the level of the individual oligosaccharides.

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CHAPTER FIVE

Reversed-phase ion-pair UPLC-MS for fingerprinting low-molecular-weight heparins

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Abstract: Heparin is a complex mixture of sulfated linear carbohydrate polymers. It is widely used as an antithrombotic drug, though it has been shown to have a myriad of additional biological activities. Heparin is often partially depolymerized to decrease the average molecular weight, as it has been shown that low molecular weight heparins (LMWH) possess more desirable pharmacokinetic and pharmacodynamic properties than unfractionated heparin (UFH). Due to the prevalence of LMWHs in the market and the emerging availability of generic LMWH products, it is important that analytical methods be developed to ensure the drug quality. This work explores the use of tributylamine (TrBA), dibutylamine (DBA), and pentylamine (PTA) as ion-pairing reagents in

conjunction with acetonitrile and methanol modified mobile phases for reversed-phase ion-pairing ultraperformance liquid chromatography coupled to mass spectrometry (RPIP-UPLC-MS) for fingerprint analysis of LMWH preparations. RPIP-UPLC-MS fingerprints are presented and compared for tinzaparin and enoxaparin.

5.1 Introduction

Heparin is a highly charged linear polysaccharide used clinically as an anticoagulant. The anticoagulant activity of heparin is derived from a specific pentasaccharide sequence with an uncommon 3-*O* sulfation of the glucosamine residue.¹
² The heparin biopolymer also contains significant regions that do not possess anticoagulant activity, but mediate other biological processes through protein binding. In addition to heparin's interaction with antithrombin III, it has been shown to act on other regulatory proteins such as growth factors³ and cytokines⁴ which are involved in processes such as angiogenesis and tumor metastasis.

Porcine intestinal mucosa heparin ranges in molecular weight from 3 to 40 kDa with an average chain length of 15 kDa.⁵ When used as the intact biopolymer, it is referred to as unfractionated heparin or UFH. The direct administration of UFH can lead to undesired side effects such as heparin-induced thrombocytopenia and osteoporosis.^{6,7} To minimize these side effects and increase the bioavailability of the drug, a low molecular weight heparin (LMWH) preparation is often prescribed. LMWHs are prepared from UFH through partial depolymerization using either heparin degrading enzymes or chemically via β -elimination, reductive deamination or oxidation using a

Fenton-type reaction.⁸ Differences in the selectivity of the various depolymerization strategies, and the structural modifications they introduce, produce a distinct oligosaccharide composition in the resulting LMWH.

Two common LMWH preparations are tinzaparin, sold as Innohep, and enoxaparin, sold in Europe as Clexane and in USA as Lovenox. Tinzaparin is prepared through the action of the heparin lyase enzyme heparinase I which selectively cleaves the biopolymer between GlcNS and IdoA(2S) residues through a β -elimination reaction.⁹ Enoxaparin is prepared through chemical β -elimination, where the UFH is reacted to form benzyl esters with the uronic acid carboxylate groups and then treated with base to facilitate β -elimination.¹⁰ The chemical β -elimination method does not share the specificity of the enzymatic depolymerization. In addition to reducing the average length of the heparin chains, these depolymerization result in structural modifications to the resulting oligosaccharides. While the enzymatic and chemical β -elimination reactions form a double bond in the non-reducing end uronic acid residue,¹¹ only the chemical β -elimination process can also generate a bicyclic 1,6-anhydrosugar side-product introducing additional heterogeneity into the enoxaparin preparation. Monosaccharide analysis of enoxaparin reported that 4.4 % of the aminosugars contained the 1,6-anhydro structure¹² and overall 15-25% of the oligosaccharide chains will be terminated by this modification at the reducing end.¹³ Structures of typical depolymerization products are illustrated in section 1.4. Tinzaparin and enoxaparin also differ in their molecular weight distributions as illustrated by the size-exclusion chromatography (SEC) profiles for tinzaparin and enoxaparin shown in Figure 5.1A and 5.1B, respectively. Tinzaparin

contains a greater fraction of higher molecular weight oligosaccharides and has a reported average molecular weight of 8.3 kDa. Although enoxaparin has a lower average molecular weight of 5.3 kDa, it also has a greater degree of structural heterogeneity.¹⁴ Others report average molecular weights for tinzaparin and enoxaparin to be 6.7 kDa and 4.0 kDa, respectively,¹⁵ suggesting variation in samples produced by "identical" preparations or in the technique used for molecular weight determination.

Analytical methods capable of profiling heparin preparations must provide the ability to detect possible abnormalities such as increased or decreased levels of sulfation, differences in the oligosaccharide size distributions or composition, and possible contamination. Unlike most drugs, which are chemically synthesized or produced through well-defined biological expression systems, pharmaceutical heparin is currently derived from porcine intestinal mucosa. The need for improved quality assurance methods for heparin was aptly demonstrated by the 2008 world-wide heparin crisis that resulted from contamination of UFH with semi-synthetic over-sulfated chondroitin sulfate (OSCS).^{16, 17} Additionally, as generic LMWH drugs are introduced, it is important to monitor quality and consistency between manufacturers and lots. The goal of this work is to develop a fast and reproducible method for fingerprinting LMWHs to ensure drug quality and integrity and to facilitate the development of novel LMWH preparations for indications other than thrombosis.

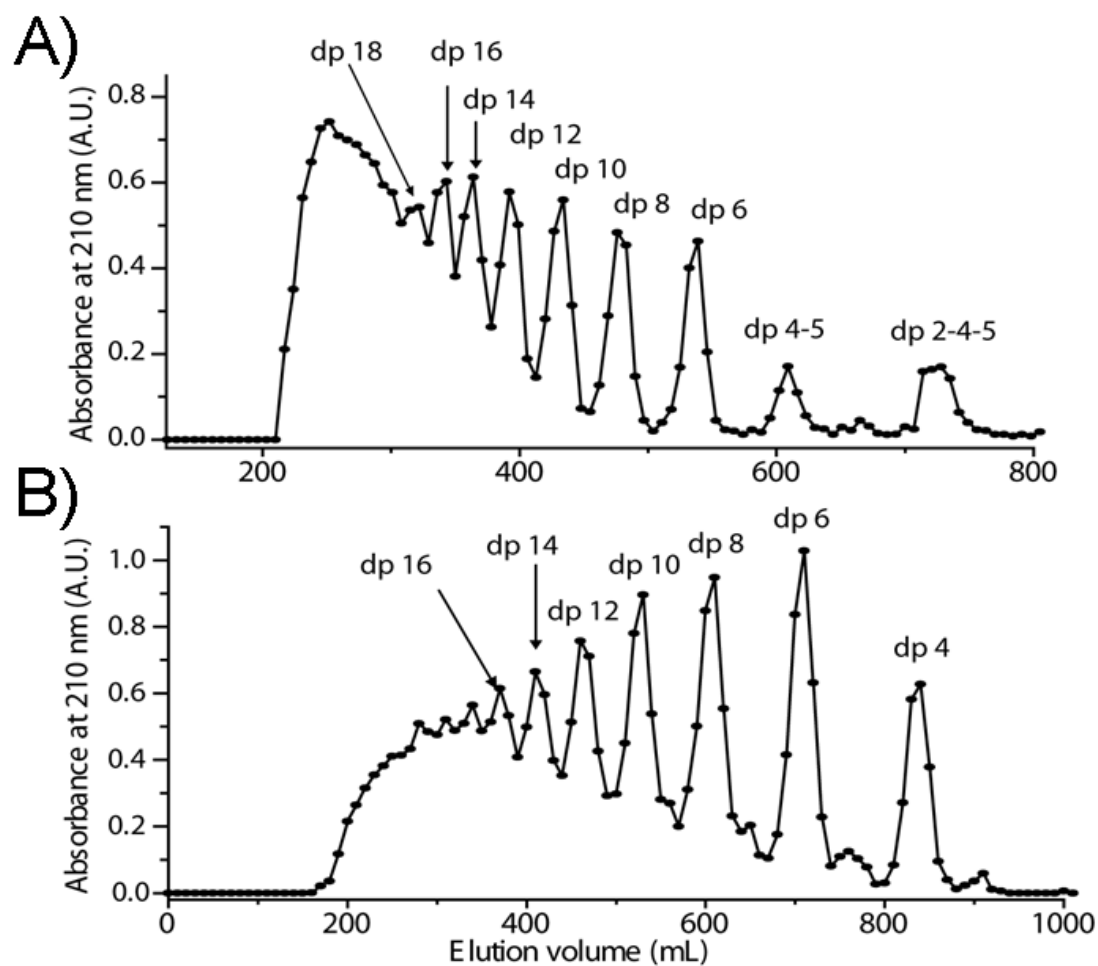


Figure 5.1. SEC profiles of LMWHs A) tinzaparin, B) enoxaparin. The abbreviation dp signifies depolymerization product indicating the number of monosaccharides per chain.

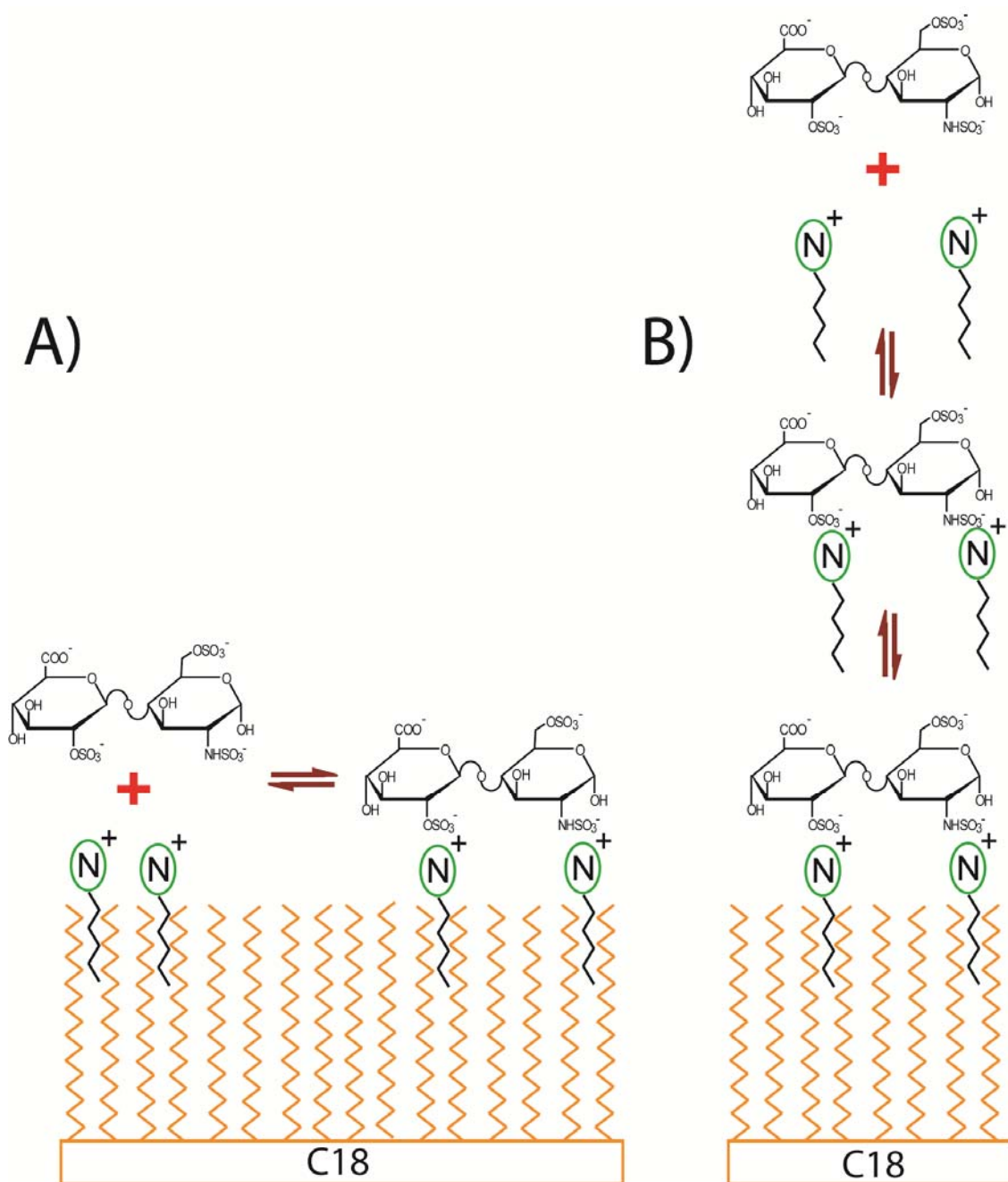


Figure 5.2. Illustration of the RPIP separation mechanisms involving the charged analyte (here a heparin disaccharide), IPR and C18 stationary phase; A) dynamic coating of a C18 column with the IPR which separates analytes via an ion-exchange mechanism, B) formation of an ion-pair in the mobile phase which migrates to the stationary phase.

An increasingly popular method for LMWH compositional analysis is reversed-phase ion-pairing liquid chromatography coupled to mass spectrometry (RPIP-LC-MS). As illustrated in Figure 5.2, RPIP-LC separations use lipophilic ions or ion-pairing reagents (IPRs) as mobile phase modifiers to facilitate retention of charged hydrophilic molecules on a hydrophobic (typically C18) stationary phase.¹⁸⁻²⁰ The separation mechanism for RPIP has been shown to result from a dynamic equilibrium between charged analytes, the IPR, and the hydrophobic stationary phase.^{21, 22} Figure 5.2 illustrates the two main mechanisms postulated for RPIP separations. Figure 5.2A represents the dynamic coating of the stationary phase with the IPR. In this mechanism, the stationary phase is coated with the IPR and the charged analyte interacts with the IPR through an ion-exchange mechanism. The extent of the stationary phase coating is dependent on the percent of organic modifier in the mobile phase. The degree of interaction between the coated stationary phase and the analyte is influenced by extent and distribution of charges on the analyte. Figure 5.2B shows the formation of an ion-pair between the charged analyte and IPR in the mobile phase and the subsequent interaction of the ion-pair with the C18 stationary phase. This mechanism is dominated by the interaction between the analyte and IPR in solution and is heavily influenced by other ions in solutions which can co-compete as ion-pairs. The percent of organic modifier in the mobile phase has a different effect on the separations governed by these competing mechanisms. In the mechanism shown in Figure 5.2A, high concentrations of the organic phase reduce the extent of IPR coating on the column. In the mechanism illustrated in Figure 5.2B, increasing the organic concentration in the mobile phase favors ion-pair

formation in solution but decreases the degree of interaction between the ion-pair and the stationary phase. In both mechanisms the steric bulk of the IPR and accessibility of the charged groups on the analyte determines the stability of the interaction between the analyte and IPR, as observed for the chromatographic resolution of isomeric disaccharides.²² The existence of two competing separation mechanisms and the dependence of the separation on the structures of both the IPR and analytes make method development for RPIP-LC more complicated than for traditional reversed-phase separations.

RPIP-LC has shown utility primarily in determinations of heparin disaccharide composition,²³⁻²⁶ but separations of larger heparin-derived oligosaccharides have been reported as well.²⁷⁻³² For heparin analysis protonated alkylamines are typically used as IPRs along with a C18 stationary phase. This type of separation provides several benefits. Various different IPRs can be used with a single C18 column, depending on the desired selectivity. This means that a single column can provide an array of separation selectivities by simply changing the IPR in the mobile phase. Another advantage is the volatility of many alkylamine IPRs making the separation easily amendable to detection by mass spectrometry. Mass spectrometry can provide critical characterization information regarding oligosaccharide length and the extent of sulfation and acetylation in addition to identifying modifications resulting from the depolymerization process such as the 1,6-anhydrosugars of enoxaparin.

The RPIP-LC-MS analysis of LMWH is not without its challenges. One of the greatest challenges is the inherent complexity of the LMWH materials. LMWHs are not

only polydisperse, covering a broad range of molecular weights, but also contain a large number of structural isomers at each chain length that can have similar retention times. Ultraperformance Liquid Chromatography (UPLC) offers a several advantages over traditional HPLC instruments. UPLC instruments are able to operate at higher pressures than regular HPLC systems and thus facilitate the use of columns with smaller particles increasing mass transfer while reducing the band broadening contributions of eddy diffusion.^{33, 34} This results in sharper chromatographic peaks, an especially important consideration when separating complex mixtures of structural isomers, and gives rise to shorter separation times which increase sample throughput.

This work explores the application of RPIP-UPLC-MS for fingerprinting the LMWH preparations tinzaparin and enoxaparin. Mobile phase and IPR selection are evaluated in terms of chromatographic resolution, analysis speed and retention time reproducibility. The LMWHs tinzaparin and enoxaparin were selected for this study because of their contrasting preparation methods and differing molecular weight distributions as shown by the preparative scale SEC profiles in Figure 5.1. Since tinzaparin is a chemically simpler mixture, it was used for method development even though it has a greater fraction of high molecular weight oligosaccharides. The separation parameters producing the best results for tinzaparin were then tested with enoxaparin, which has greater chemical heterogeneity.

5.2 Methods and Materials

5.2.1. Material and reagents. Acetonitrile (optima grade), methanol (optima grade) and glacial acetic acid (USP grade) were purchased from Fisher-Scientific (Pittsburgh, PA). HPLC-grade water was from Burdick and Jackson (Morristown, NJ). Tributylamine (TrBA) (purity $\geq 99\%$) and *n*-amylamine (PTA) (purity $> 99\%$) were purchased from Acros Organics (Fair Lawn, NJ). Dibutylamine (DBA) (purity $> 99\%$) was purchased from Sigma-Aldrich (St. Louis, MO). Enoxaparin was purchased from the U.S. Pharmacopeia (Rockville, MD) and tinzaparin was supplied by LEOPharma (Ballerup, Denmark). The Clexane (Lot 1LC18, 8000 u.i. aXa/0.8 mL) was obtained from Sanofi Aventis (Paris, France). E.D.Q.M. sodium enoxaparin (certified by the European Directorate for the Quality of Medicines & HealthCare) was acquired through the European Pharmacopoeia (Strasbourg, France).

5.2.2. Size-exclusion chromatography profiling of LMWH. SEC separations were performed using a Bio-gel P10 column (2.6 cm x 160 cm) with a flow rate of 0.5 mL/min and a mobile phase of consisting of 150 mM NaCl and 50 mM phosphate adjusted to pH 7.2. The chromatogram was obtained by monitoring UV absorbance at 210 nm.³⁵

5.2.3. UPLC separations. All high pressure chromatographic separations were performed with a 2.1 mm x 150 mm Acquity UPLC BEH C18 column with 1.7 μm particles (Waters Corporation, MA). A guard column with an identical packing was used prior to the analytical column. The column was maintained at 40 °C for all separations. Analyses were performed by injecting 20 μL of a 1 mg/mL solution of the LMWH dissolved in water. A binary solvent system was used for gradient elution. Solvent A

consisted of 95% water and 5% of the respective organic solvent, either acetonitrile (ACN) or methanol (MeOH). Solvent B consisted of 100% organic solvent. Solvents A and B both contained 10 mM of the IPR and 10 mM acetic acid.

Separations were performed using DBA, TrBA, and PTA using ACN as the mobile phase at a flow rate of 0.4 mL/min. Separations using DBA as the IPR and MeOH as the mobile phase were performed using a flow rate of 0.3 mL/min. A 10 min wash of 100% solvent B followed by an equilibration period was performed between sample injections to ensure complete equilibration of the column. Gradient profiles were optimized for each set of chromatographic conditions by maximizing chromatographic peak resolution within the constraints of the target analysis times and are summarized in Table 5.1.

5.2.4. Mass spectrometry. Total ion chromatograms (TICs) were collected using a Micromass Q-TOF micro ESI quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA). Data acquisition was accomplished using MassLynx 4.1 software. All spectra were obtained in negative ion mode using the following instrument parameters: capillary voltage, 2.6 kV; cone voltage, 24 V; source temperature, 120 °C; desolvation temperature, 250 °C; extractor voltage, 1 V; radio frequency lens, 0.5 V; interscan delay, 0.1 s; m/z range of 350 to 2000. Chromatograms were enhanced using the strip command within MassLynx.

Table 5.1. Gradient profiles used for each experiment.

IPR	Solvent B	Time (min)	% Solvent B	Chromatogram
DBA	MeOH	0	27	Figure 5.3A
		1	27	
		16	47	
		40	67	
DBA	MeOH	0	26	Figure 5.3B
		1	26	
		100	69	
DBA	ACN	0	18	Figure 5.5A
		1	18	
		32	28	
		40	34	
TrBA	ACN	0	34	Figure 5.5B
		1	34	
		40	61	
PTA	ACN	0	5	Figure 5.5C
		1	5	
		10	13	
		40	69	
PTA	ACN	0	5	Figure 5.6A
		1	5	
		5	13	
		20	32	
PTA	ACN	0	5	Figure 5.6B
		1	5	
		70	21	
		100	29	

5.3 Results and Discussion

5.3.1. Mobile phase selection. Mobile phase selection is one of the most important considerations in designing a separation method. ACN and MeOH are both commonly used organic solvents for reversed-phase separations. Separations of heparin oligosaccharides using RPIP typically use ACN as the mobile phase, but results for the separation of isolated oligosaccharides using MeOH in conjunction with DBA have also been reported.³⁶ Figure 5.3 shows the UPLC-MS results obtained for tinzaparin using DBA and MeOH in a 40 min separation. Because of the higher viscosity of MeOH-water mixtures, the maximum flow rate that could be used with MeOH was 0.3 mL/min at 40 °C. Although a lower back pressure (and therefore a faster flow rate) could be achieved using a 10 cm column, to obtain the best resolution for these complex LMWH samples the greater resolving power of a longer column is required.

It is interesting to note the pattern in Figure 5.3 of sets of two intense peaks for each oligosaccharide length along with many smaller peaks. These pairs of intense peaks observed for each oligomer length correspond to the fully sulfated structure and to oligosaccharides containing one sulfate less than the maximum number. The prominent peaks are labeled using the $\Delta X,Y,Z$ scheme in which the Δ indicates an unsaturated bond in the uronic acid residue of the non-reducing end of the newly formed oligosaccharide, X is the number of monosaccharide units comprising the oligosaccharide, Y is the number of sulfate groups and Z is the number of *N*-acetyl groups. It is important to note that although one fully sulfated hexasaccharide ($\Delta 6,9,0$) is expected, 9 sulfation isomers are possible for the corresponding $\Delta 6,8,0$ hexasaccharide. In addition to the heterogeneity

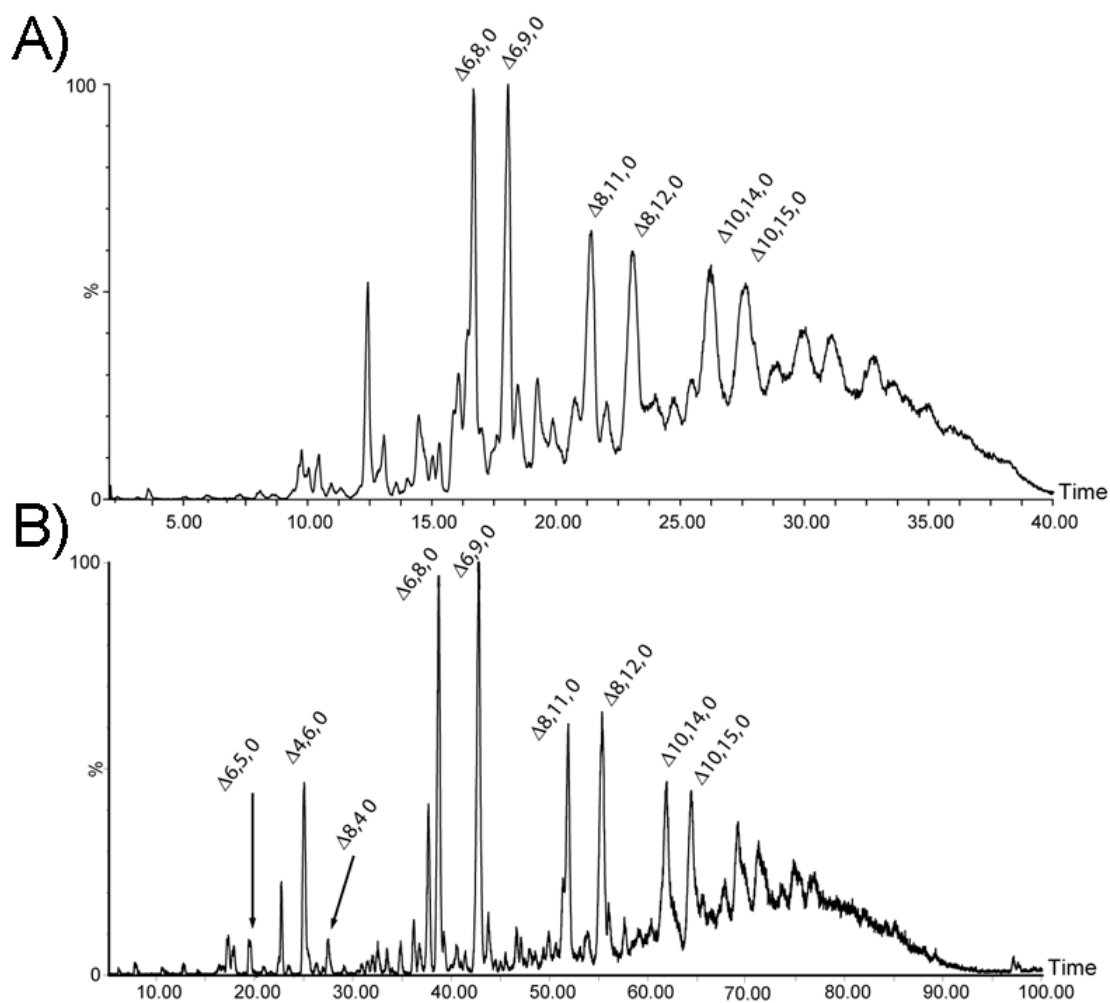


Figure 5.3. RPIC-UPLC-MS chromatograms measured for tinzaparin using 10 mM DBA and MeOH as the organic mobile phase with analysis times of (A) 40 min and (B) 100 min.

arising from sulfation isomers, additional complexity results from the presence of uronic acid epimers, as iduronic acid and glucuronic acid are both possible in the heparin biopolymer, and the presence of α and β anomers at the oligosaccharide reducing end.^{27,}

37

To evaluate whether increasing the separation time would significantly improve the resolution of the separation, the analysis time was extended to 100 min (Figure 5.3B). This chromatogram shows a greater degree of separation of isomeric peaks for smaller oligosaccharides with good resolution up to the octasaccharide peaks. Peaks corresponding to $\Delta 6,5,0$, $\Delta 4,6,0$, and $\Delta 8,4,0$ in Figure 5.3B have a degenerate charge of -8 and are baseline resolved. However, both the 40 and 100 min chromatograms measured using DBA and MeOH have poor resolution in the later region of the chromatogram where the larger oligosaccharides are expected to elute. Although this method shows promise for the analysis of heparin-derived oligosaccharides smaller than decamers (e.g ultra-LMWHs), it is not suitable for LMWH fingerprinting because of the broad chromatographic peaks obtained for the larger oligomers.

5.3.2. Comparison of DBA, TrBA, and PTA separations. IPRs were chosen based on their relative hydrophobicities. Figure 5.4 presents a comparison of chromatograms measured for tinzaparin using an identical linear gradient and solvent conditions for each of the IPRs tested. This figure indicates that tinzaparin was retained less using PTA and was most highly retained by TrBA.

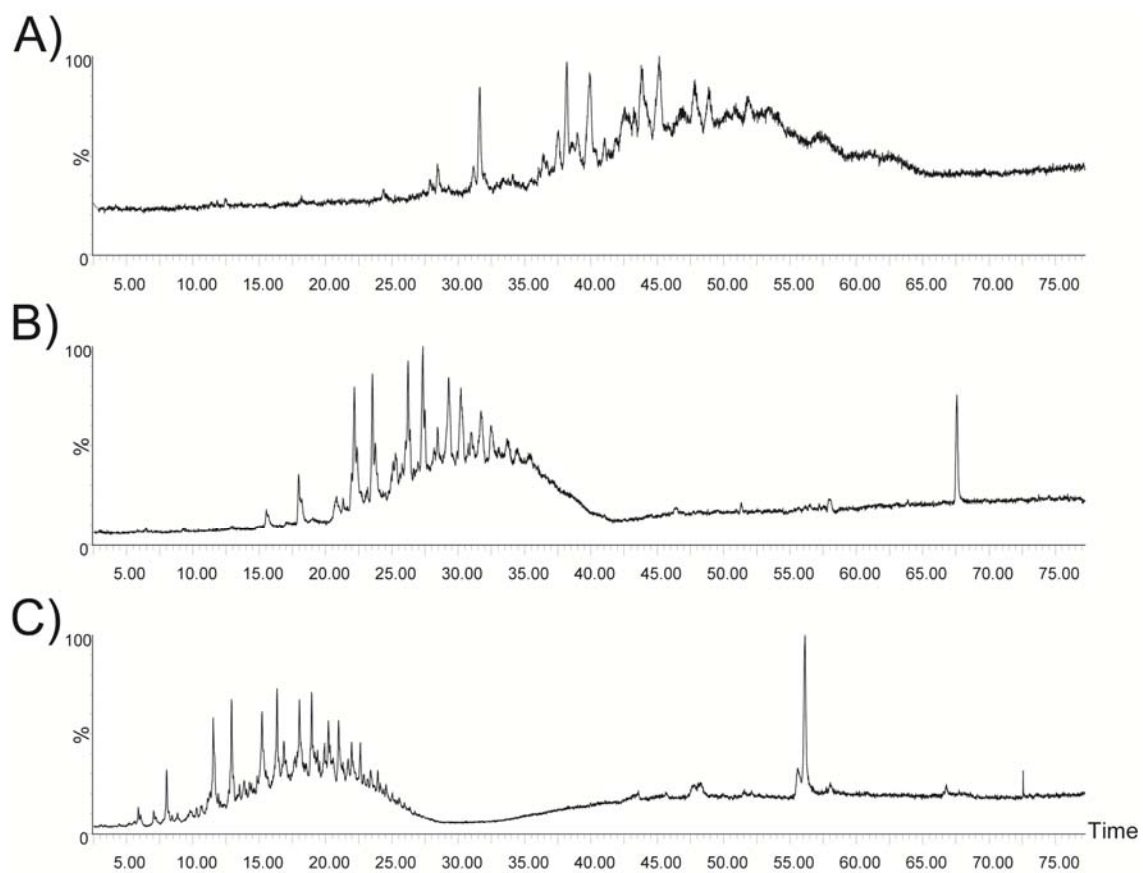


Figure 5.4. Identical separation conditions for the separation of tinzaparin where IPR used in A) TrBA, B) DBA, and C) PTA.

Due to their different retention properties it was necessary to develop a slightly different gradient method for each IPR (Table 5.1). The reported chromatograms represent separations optimized in terms of chromatographic peak resolution for each IPR. To evaluate the chromatographic resolution, relative MS ionization efficiency and the oligosaccharide size range that can be detected using these IPRs, separation methods were developed for each IPR while keeping all other variables constant. Figure 5.5 compares the 40 min separations obtained for tinzaparin using 10 mM of each IPR.

The chromatogram obtained for tinzaparin using 10 mM DBA and ACN is presented in Figure 5.5A. This separation shows improvement over the 40 min separation using DBA and MeOH (Figure 5.3A). In addition to its different solvating properties, a higher flow rate can be used with ACN which improves chromatographic resolution.³⁸ This chromatogram shows resolution up to the fully sulfated tetradecasaccharide and reveals a peak pattern similar to that observed with DBA and MeOH (Figure 5.3A). Although the separation of tinzaparin using DBA and ACN yields good chromatographic resolution of the early eluting peaks, peak intensity trails off at the end of the separation. The poor MS sensitivity obtained for the largest oligomers limits the applicability of this separation as a fingerprinting method.

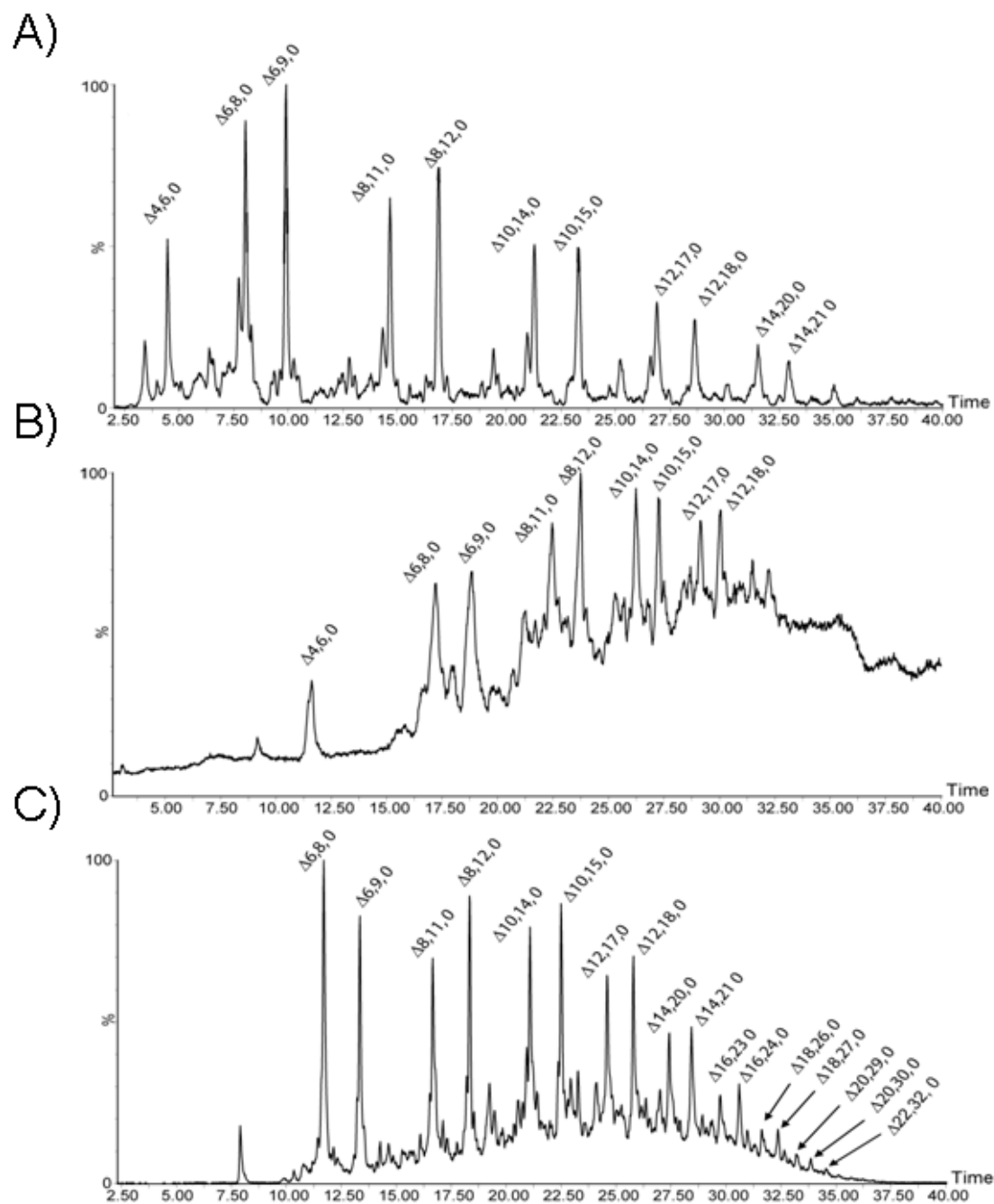


Figure 5.5. Optimized 40 min RPIP-UPLC-MS separations of tinzaparin using ACN as the organic mobile phase and 10 mM A) DBA, B) TrBA, and C) PTA in both the aqueous and organic phases.

TrBA, which has been shown to work well for heparin disaccharide analyses,^{21, 39} was also evaluated as an IPR for the ACN separation of tinzaparin (Figure 5.5B). The additional butyl group in TrBA makes it more hydrophobic than DBA and adds steric bulk which could impact the selectivity of the separation. Of the IPRs tested, TrBA shows the poorest performance in the tinzaparin separation (Figure 5.5B). The resolution breaks down at an even lower oligosaccharide size range than observed for DBA and MeOH. One of the main problems with TrBA is that it interacts more strongly with the C18 stationary phase than the other IPRs tested. As a result, higher molecular weight oligosaccharides bearing greater numbers of negative charges interact strongly with the TrBA sorbed onto the hydrophobic stationary phase and a higher organic composition mobile phase is required for their elution. When the organic composition of the mobile phase reaches ~56% ACN in the chromatogram shown in Figure 5.5B, it is no longer possible to detect individual oligosaccharides above the noise in the mass spectrum. At this mobile phase composition it is likely that TrBA is being eluted from the column along with all of the remaining sorbed heparin oligosaccharides. As a result, ionization is suppressed and individual oligosaccharide peaks are not detected against the high background.

The chromatogram obtained using 10 mM PTA and ACN is shown in Figure 5.5C. In the PTA molecule, the two butyl side chains of DBA have been replaced by a single pentyl chain. As indicated by the chromatograms in Figure 5.4, PTA is the least hydrophobic of the IPRs tested and should also have the lowest amount of steric

hindrance in its interactions with the heparin oligosaccharides. In contrast to the separations developed with TrBA and DBA, even the largest oligosaccharides exhibit good resolution and ionization using PTA. This separation method allows clear distinction of the pattern of two intense peaks for each oligosaccharide length from the hexasaccharide up to the 20 residue oligomer (Figure 5.5C). This is important in LMWH fingerprinting as differences between preparations can be subtle and most prevalent in the larger oligosaccharides. The oligosaccharide size range observed in this tinzaparin separation correlates well with the distribution observed in the SEC chromatogram in Figure 5.1.

5.3.4. Analysis time. Time is an important aspect of any analysis method. To examine the impact of separation time, chromatograms were measured over 20 (Figure 5.6A) and 100 min (Figure 5.6B) for tinzaparin using PTA and ACN. Although the peak resolution in Figure 5.6A is poorer than that obtained in 40 min (Figure 5.5C), this chromatogram still exhibits the pattern of two intense peaks for each oligomer length characteristic of the tinzaparin sample. Doneanu et al. also reported a 20 min positive-ESI UPLC-MS separation for tinzaparin with PTA as the IPR but used 75% ACN with 50 mM HFIP as mobile phase B.²⁸ Compared with Figure 5.6A, their positive mode TIC has poorer resolution of the two prominent peaks than we observe for each oligosaccharide length. In addition to better peak resolution, our 20 min separation also appears to provide better MS intensity for the larger oligosaccharides beyond dp12 than reported for the positive ESI method of Doneanu et al.²⁸

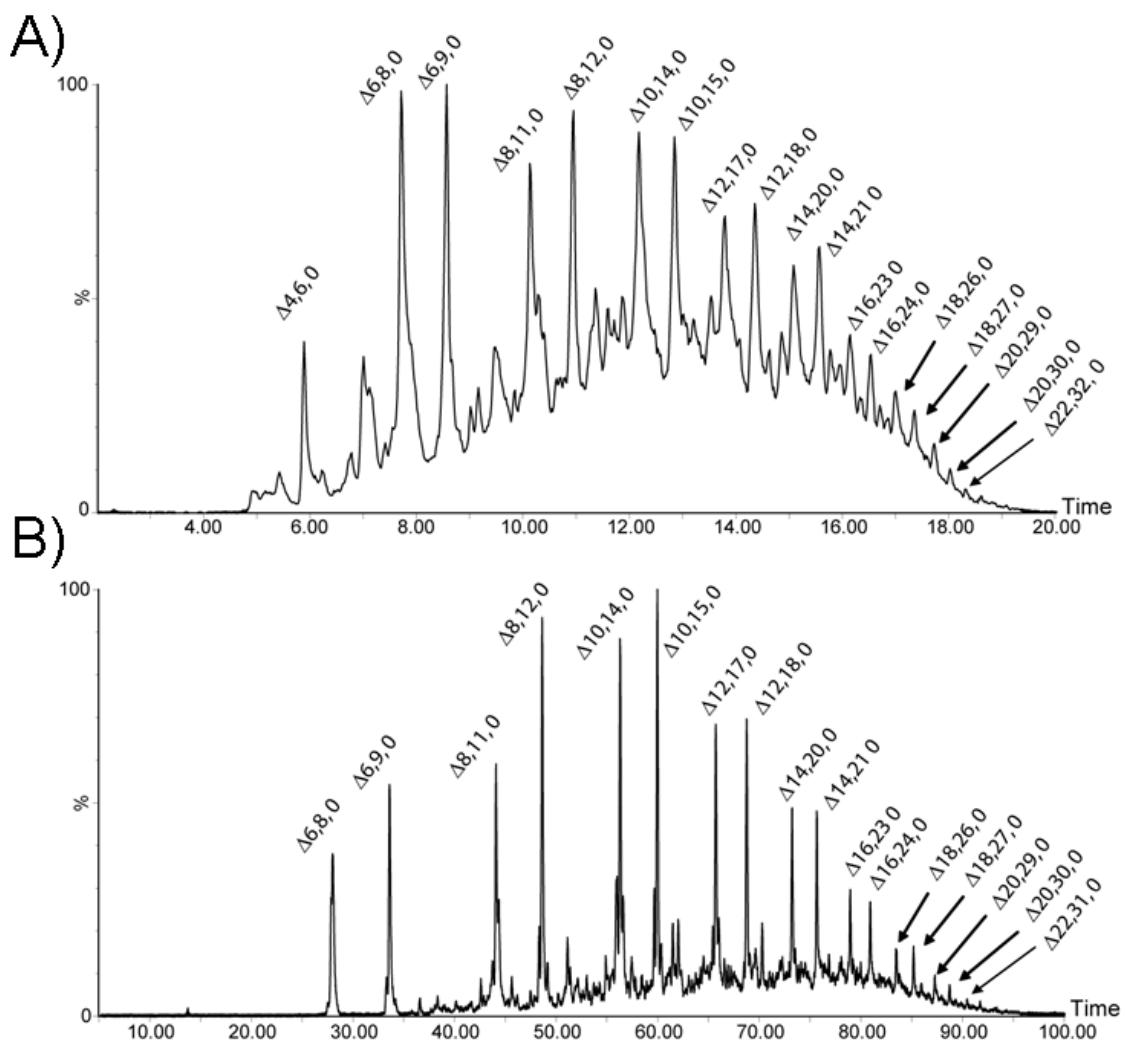


Figure 5.6. RPIP-UPLC-MS separation of tinzaparin using 10 mM PTA and ACN as the organic mobile phase with analysis times of A) 20 min and B) 100 min.

The result of extending the PTA separation time to 100 min is presented in Figure 5.6B. This chromatogram shows excellent isomer separation as well as a clear pattern of two intense peaks for each oligosaccharide length up to the 22 mer, though the early eluting peaks exhibit poorer resolution than was obtained in the 100 min separation with DBA and MeOH (Figure 5.3B). The 100 min chromatogram (Figure 5.6B) naturally also has superior resolution than the 40 min separation (Figure 5.5C). Although the increased analysis time is likely not needed for routine fingerprinting, the longer separation could be useful for samples where increased isomer resolution is required, for example in the detailed characterization of suspect samples.

5.3.5. Reproducibility. It is critical that a chromatographic method designed for quality assurance applications, such as fingerprinting of LMWHs, produces retention times with a high degree of reproducibility. Because the PTA and ACN separation method gave the best resolution over the widest oligosaccharide length range, retention time reproducibility was evaluated for selected peaks across the 40 min chromatogram (Figure 5.5C). Average retention times with percent relative standard deviation (%RSD) values are reported in Table 5.2 along with the TIC intensity of each peak and its %RSD. No significant drift in the RSDs are observed as the retention time increases. Even with the much greater complexity of the tinzaparin sample and its longer elution profile, the results in Table 5.2 are in good agreement with the RSD values we reported for the RPIP-UPLC-MS separation of heparin disaccharide standards using TrBA and ACN.⁴⁰

The %RSD for the TIC peak intensities also suggests good reproducibility of peak heights suggesting that, using the current instrument configuration, differences in peak height as small as 5% can be detected.

5.3.6. Mass spectral assignments and interpretation. Examples of the negative ESI mass spectra used to make the oligosaccharide assignments indicated in the labeled chromatograms are shown in Figure 5.7. The example spectra were taken from the tinzaparin chromatogram in Figure 5.5C. It is relatively straight forward to use mass spectra to assign the identities of abundant oligosaccharides with unique masses. The key to assigning these mass spectra is accounting for the characteristic mass differences for sulfate loss (-80 Da) and IPR adduction (+87 Da for PTA). For example, the mass spectrum in Figure 5.7A for the well-resolved chromatographic peak at 7.90 min in Figure 5.5C can be assigned to the fully sulfated tetrasaccharide $\Delta 4,6,0$. The doubly charged molecular ion at m/z of 576.24 is present in moderate abundance and loss of sulfate is indicated by the doubly charged peaks at m/z 536.22, 496.24, and 456.24 which arise from the loss of 1, 2, and 3 sulfate groups, respectively. A singly charged low intensity peak can be observed at 672.35 corresponding to the completely desulfated tetrasaccharide.

The assignment process becomes increasingly more difficult as the oligosaccharide length increases. The mass spectrum of the chromatographic peak designated $\Delta 8,12,0$ eluting at 18.24 min, shown in Figure 5.7B, is substantially more complex. In this mass spectrum a molecular ion is not detected and both -2 and -3 charge

Table 5.2. Average retention times and total ion counts with percent relative standard deviations (RSDs) for 5 separate Tinzaparin separations using the 40 min PTA and ACN method shown in Figure 5.5C.

Average Retention Time (min)	% RSD	Total Ion Count	% RSD
8.38	0.46	5476	4.89
14.05	0.37	10446	3.65
19.14	0.29	10308	4.41
23.43	0.23	8412	2.56
26.73	0.22	6570	3.62
29.4	0.21	5011	2.37
31.54	0.18	3754	2.10

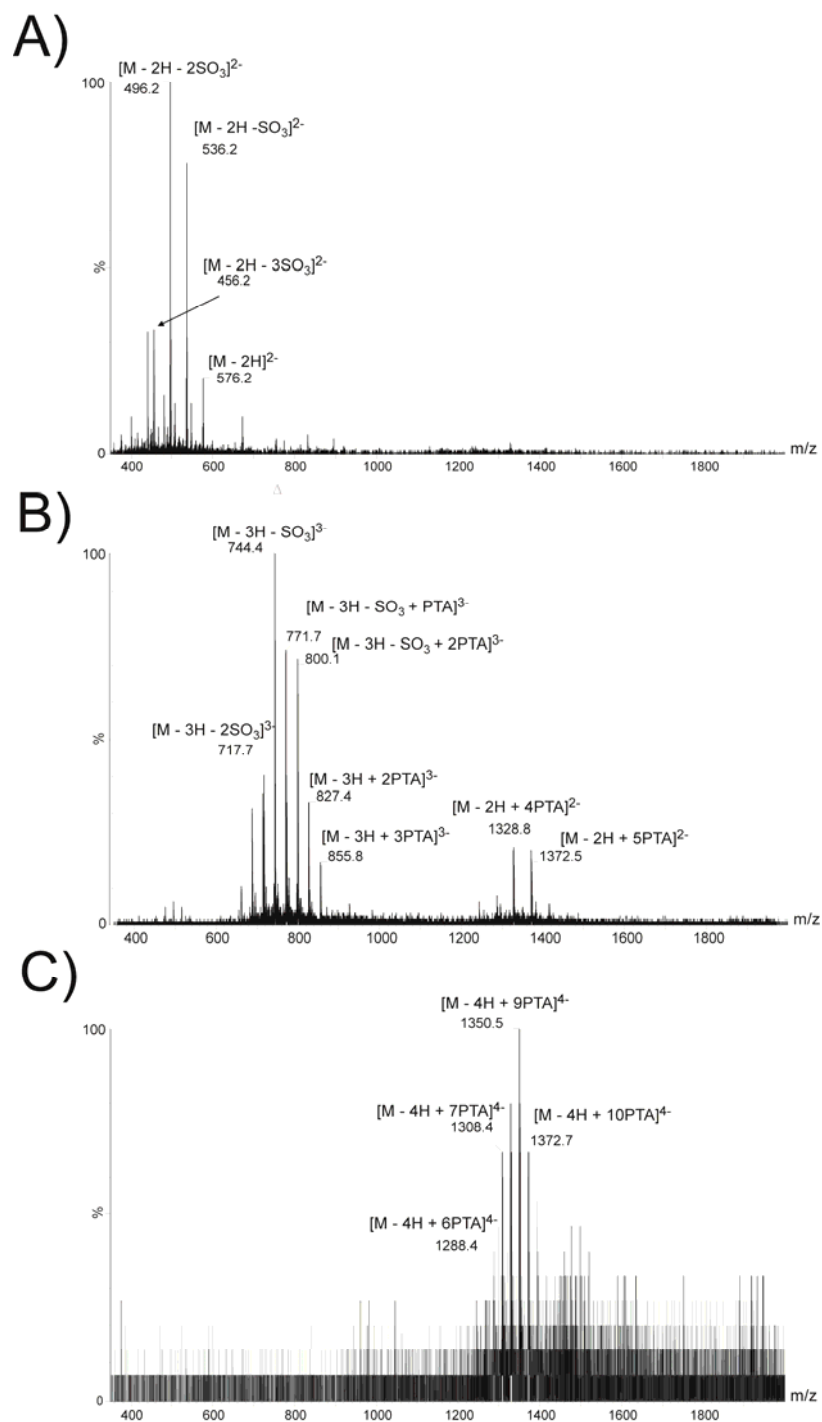


Figure 5.7. Mass spectra corresponding to chromatographic peaks from the optimized 40 min separation using PTA and ACN assigned as A) $\Delta 4,6,0$ and B) $\Delta 8,12,0$, and C) $\Delta 16,24,0$.

states are observed, further complicating the analysis. Peaks corresponding to doubly charged ions with adducts of 4 and 5 PTA molecules have m/z values of 1328.83 and 1372.41, respectively. Triply charged ions with 2 and 3 PTA adducts have m/z values of 827.38 and 855.76. Triply charged peaks corresponding to ions with PTA adduction and sulfate loss can be observed at m/z values of 800.08, 771.70, and 744.37. Although it is more complex than assigning the tetrasaccharide mass spectrum in Figure 5.7A, due to the unique masses of the PTA adducts and sulfate losses it is still possible to determine the m/z of the parent oligosaccharide. MS analysis of even larger oligosaccharides yield only PTA adducts of the parent ion observed in higher charge states, as seen in the MS spectrum for $\Delta 16,24,0$ in Figure 5.7C. These oligosaccharides can be assigned by comparing predicted m/z values to those with varying number of IPR adducts.

The analysis of less abundant oligosaccharides with non-unique masses must rely more heavily on chromatographic resolution. To illustrate this challenge, Figure 5.8 shows an extracted ion chromatogram for m/z of 913.29 which corresponds to oligosaccharides with the designation $\Delta 4,3,0$ and a single negative charge. Because heparin is highly sulfated, this oligosaccharide should be present in low abundance and is expected to elute prior to the more highly sulfated tetrasaccharides. Figure 5.8 shows chromatographic peaks corresponding to this mass over a wide range of the chromatogram. The m/z corresponding to $\Delta 4,3,0$ is also detected at the elution times of more highly sulfated parent tetrasaccharides that have experienced sulfate loss in the mass spectrometer or as a fragment produced by larger oligosaccharides with higher

charge. This makes chromatographic resolution paramount if the oligosaccharides of interest have common or non-unique masses.

Doneanu et al. reported mass spectra for a size-uniform fraction of heparin-derived hexasaccharides collected in positive-ESI-MS using PTA adduct ions enhanced by the presence of HFIP.²⁸ The work described herein employed a similar strategy focusing solely on negative-ion mass spectra. In our hands the presence of PTA adducts and ions corresponding to sulfate loss, including ions of higher charge and lower m/z , were useful in the assignment of chromatographic peaks. Although we attempted to measure mass spectra in positive mode, we were unable to achieve effective ionization without the use of HFIP. The use of HFIP as a mobile phase additive is not ideal due to its expense and the difficulty in removing trace amounts from the column and the UPLC system, especially in a laboratory such as ours where the same system is used to address a wide range of different analytical problems.

The largest oligosaccharide identified using this method was $\Delta 22,32,0$. As oligosaccharide length increases, the number of potential isomers increases dramatically. This reduces the chromatographic resolution which in turn decreases the ability of the ESI source to efficiently ionize each coeluting isomer. With newer ESI designs or alternate ionization methods it may be possible to enhance the ionization of large oligosaccharides extending the detectable molecular weight range using this separation method.

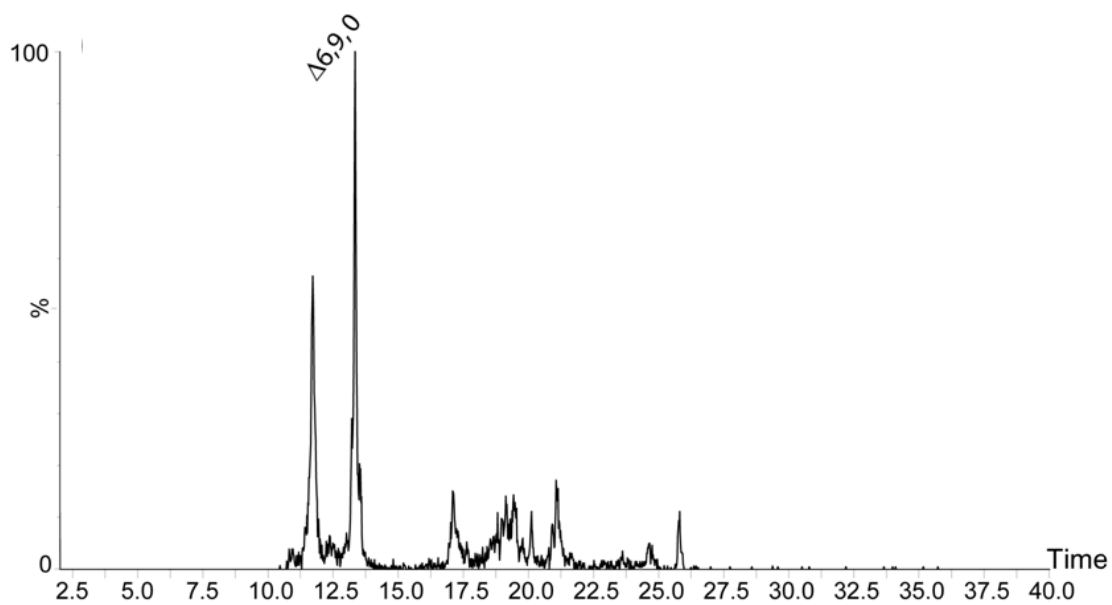


Figure 5.8. Extracted ion chromatogram for the mass of 913.29 ± 0.5 Da corresponding to the m/z of the singly charged oligosaccharide with configuration $\Delta 4,3,0$.

5.3.7. Comparison of tinzaparin and enoxaparin RPIP-UPLC-MS fingerprints. To validate this method for fingerprinting LMWH preparations, the profile of enoxaparin was measured and compared to that of tinzaparin using the optimized PTA and ACN method used in Figure 5.5C. Striking differences between the tinzaparin (Figure 5.9A) and enoxaparin (Figure 5.9B) chromatograms can be observed. The first difference is with respect to the oligosaccharide size distribution. In addition to being comprised of a higher percentage of lower molecular weight oligosaccharides, the enoxaparin chromatogram shows a greater diversity of oligosaccharides as evidenced by the larger number of peaks detected for each oligomer length. The numerous well-resolved tetra-, hexa- and octasaccharide peaks in Figure 5.9B illustrate the ability of this method to separate these components even though few peaks for these oligomer lengths are detected in the tinzaparin chromatogram (Figure 5.9A).

A second striking difference between the profiles of tinzaparin and enoxaparin is in the patterns produced for each group of depolymerization products. In the fingerprint of tinzaparin, two prominent sharp peaks that differ by a single sulfate group were detected for each even numbered oligosaccharide. Not only is this dual peak pattern not observed in the enoxaparin chromatogram, Figure 5.9B shows a unique and unusual pattern of ‘split peaks’ for each oligomer length for the oligosaccharide containing 3 sulfates per disaccharide, e.g., $\Delta 4,6,0$, $\Delta 6,9,0$, $\Delta 8,12,0$, etc. Our initial assumption was that these peaks might be due to the 1,6-anhydrosugar modifications to the saccharide reducing ends expected in enoxaparin. However, as shown in Figure 5.10, our MS data confirms that the mass spectra of the major components of these split chromatographic

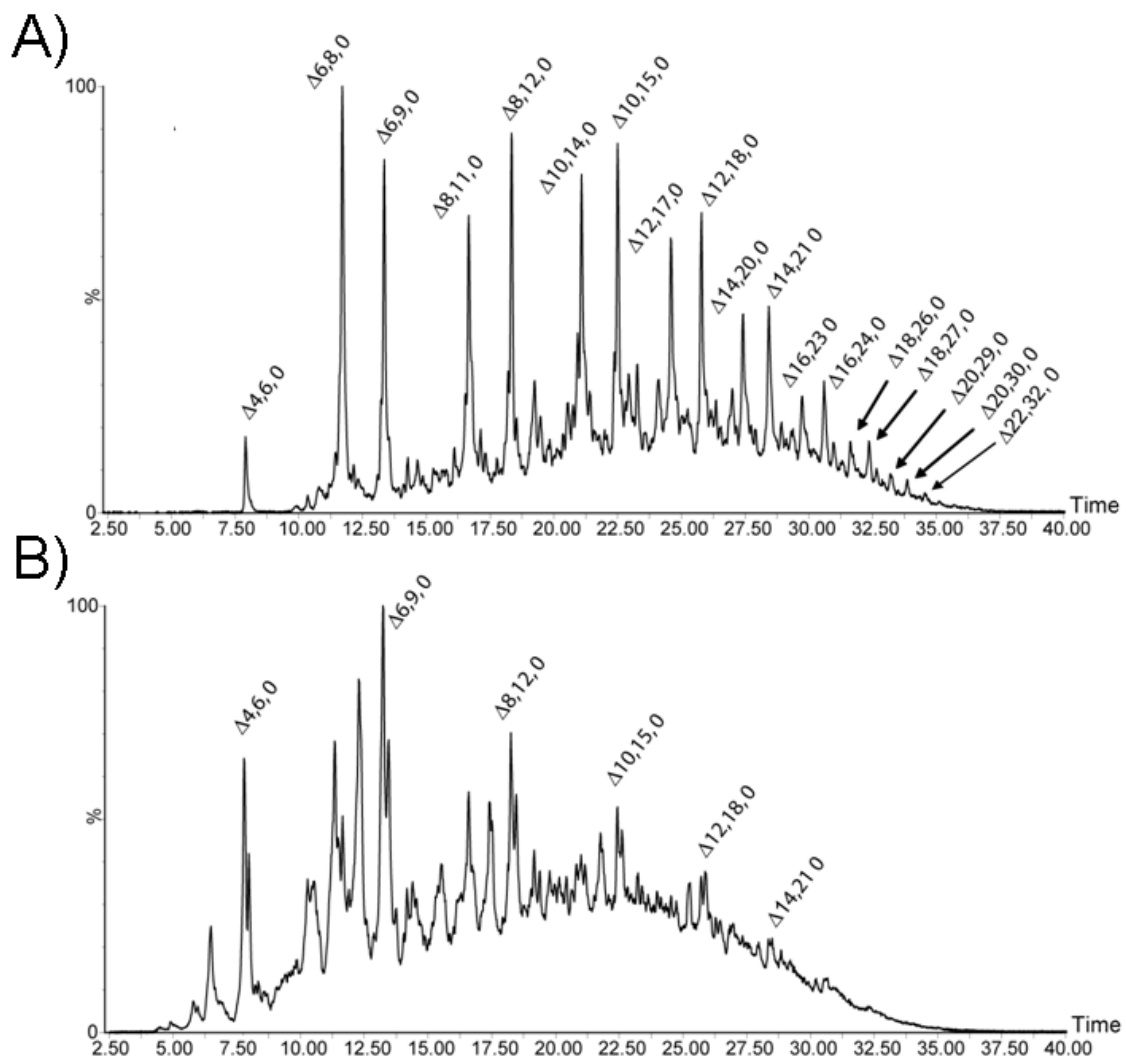


Figure 5.9. Chromatographic fingerprints of (A) tinzaparin and (B) enoxaparin with peak annotations. The separation conditions are identical to those presented in Figure 5.5C.

peaks are not a result of the 1,6-anhydro structures, but are structural isomers with identical size and sulfate content. One possibility is that these split peaks arise from oligosaccharides that contain sulfated uronic acid epimers. Bisio et al. reported more than a 5 fold increase in sulfated GlcA residues in enoxaparin compared to tinzaparin¹⁴. Work by Limitaco et al. and Ozug et al. have reported that oligosaccharides that only differ by the conformation of the uronic can be separated chromatographically.^{27, 37} Another explanation of these split peaks may be epimerization of reducing end *N*-sulfoglucosamine-6-*O*-sulfate residues to *N*-sulfomannoseamine-6-*O*-sulfate residues. This process is a well known result of the chemical depolymerization used in enoxaparin production and will also yield oligosaccharide isomers with identical molecular masses.¹²

5.3.8. Comparison of the RPIP-UPLC-MS fingerprints of Clexane and the U.S.P. and E.D.Q.M. enoxaparin reference standards. The U.S.P. and E.D.Q.M. enoxaparin reference standards were analyzed along with the commercial Clexane product to test the ability of this method to distinguish between different preparations of the same LMWH samples. These samples were analyzed using the optimized 40 min PTA and ACN separation described earlier. Only subtle differences in oligosaccharide composition are expected between a certified reference material and the released product. Visual comparison of representative chromatograms of the three samples (Figure 5.11) shows an obvious difference in the early eluting peaks in chromatogram B (U.S.P. enoxaparin). The two peaks (indicated by an X in Figure 5.11B) eluting before 6.5 min are due to small oligosaccharides and are only present in this sample. These peaks reflect a

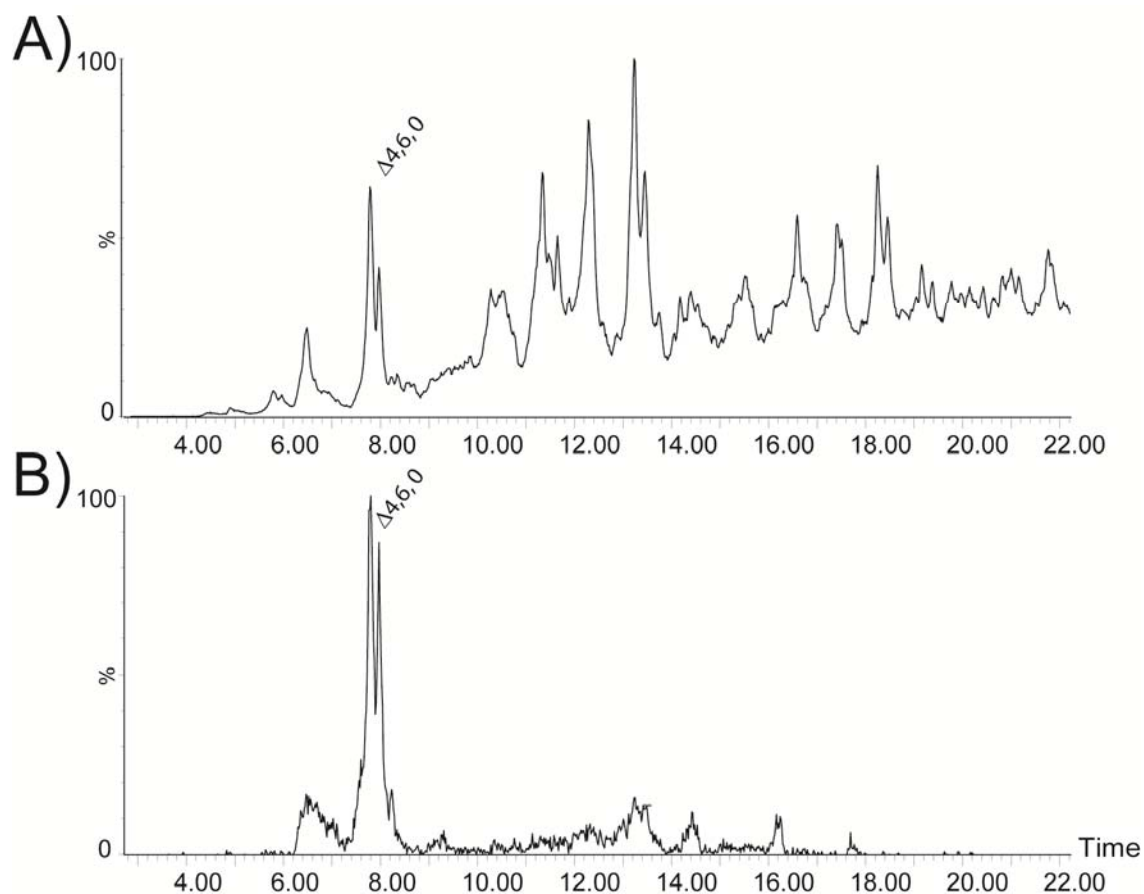


Figure 5.10. Analysis of enoxaparin using PTA and ACN. (A) Expansion of the total ion chromatogram shown in Figure 5.9B to better show the peaks of the lower molecular weight oligosaccharides. (B) Extracted ion chromatogram for mass 576.80 ± 0.5 Da corresponding to the m/z of the singly charged oligosaccharide with configuration $\Delta 4,6,0$.

difference in oligosaccharide distribution which may have resulted from differences in the conditions used in the preparation of the U.S.P. material. Another obvious difference is the greater intensity of peak 5 in Figure 5.11B compared with chromatograms A and C indicating a higher percentage of this component in the U.S.P. enoxaparin sample.

A more quantitative comparison can be obtained by analysis of average peak heights. These results are summarized in Figure 5.12 for each of the numbered peaks in the chromatograms depicted in Figure 5.11 with error bars corresponding to ± 2 standard deviations of triplicate measurements. Peak intensities were normalized to the $\Delta 6,9,0$ peak to compensate for slight differences in sample concentration or variance in injection volume. Two important differences can be noted in Figure 5.12. The data point for peak 5 for U.S.P. enoxaparin is well separated from the points representing peak 5 in E.D.Q.M. enoxaparin or Clexane, suggesting that there is a higher amount of the $\Delta 6,8,0$ oligosaccharides in the U.S.P. sample. The other major difference is between the intensity of peak 2 in the U.S.P. and E.D.Q.M. enoxaparin reference standards.

While it is not feasible to quantitatively compare differences in such complex mixtures, the current study shows that qualitative differences can be observed in different enoxaparin samples prepared using the same process. It is likely that these qualitative differences could be statistically linked to quantitative differences with a larger data training set, however such a variety of samples is not readily available and establishing this correlation is beyond the scope of the current study.

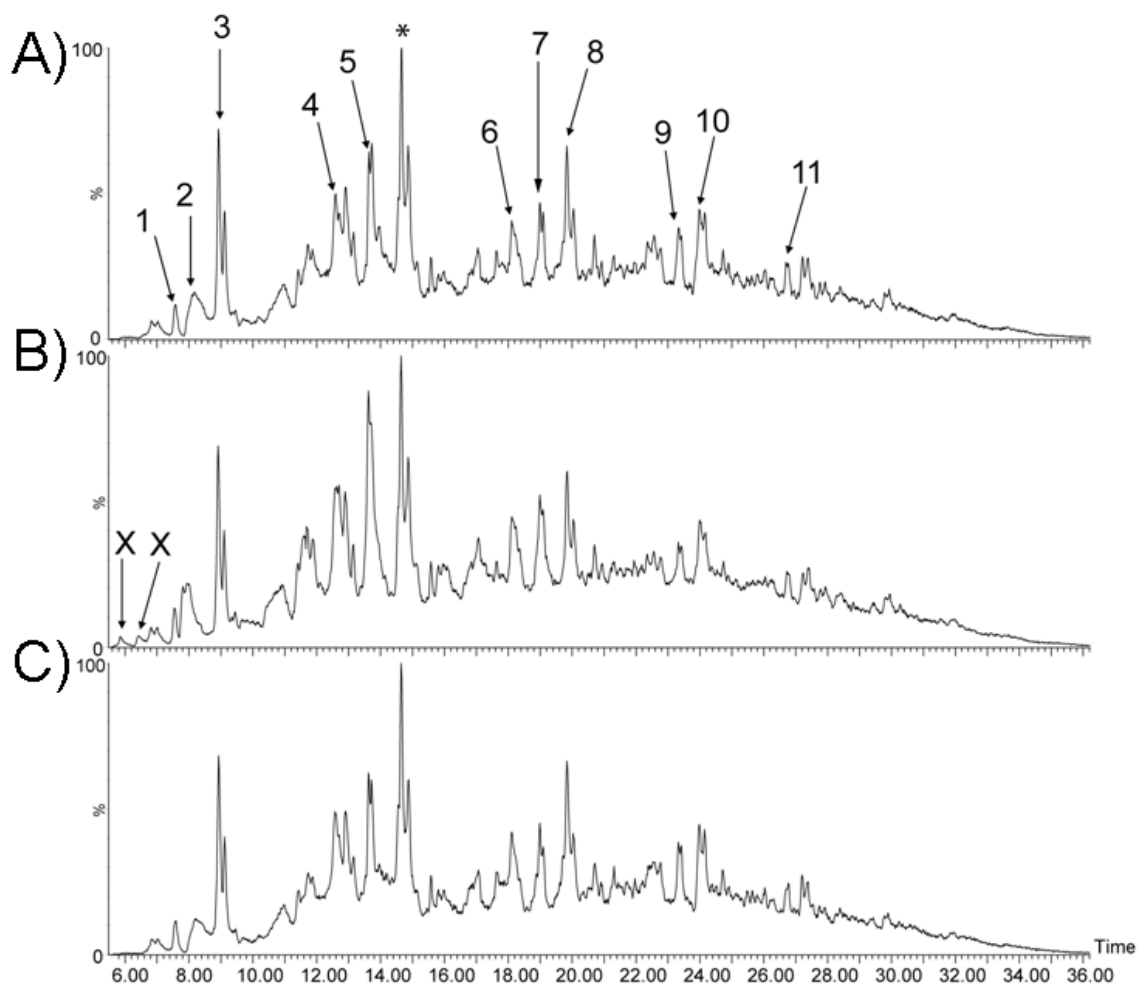


Figure 5.11. Total ion chromatograms for separations using PTA and ACN of (A) Clexane, (B) U.S.P. enoxaparin, and (C) E.D.Q.M. enoxaparin. The asterisk in (A) denotes the $\Delta 6,9,0$ peak and in (B) X identifies unique peaks not observed in the other chromatograms. The numbered peaks in chromatogram A are quantitatively compared in Figure 5.12.

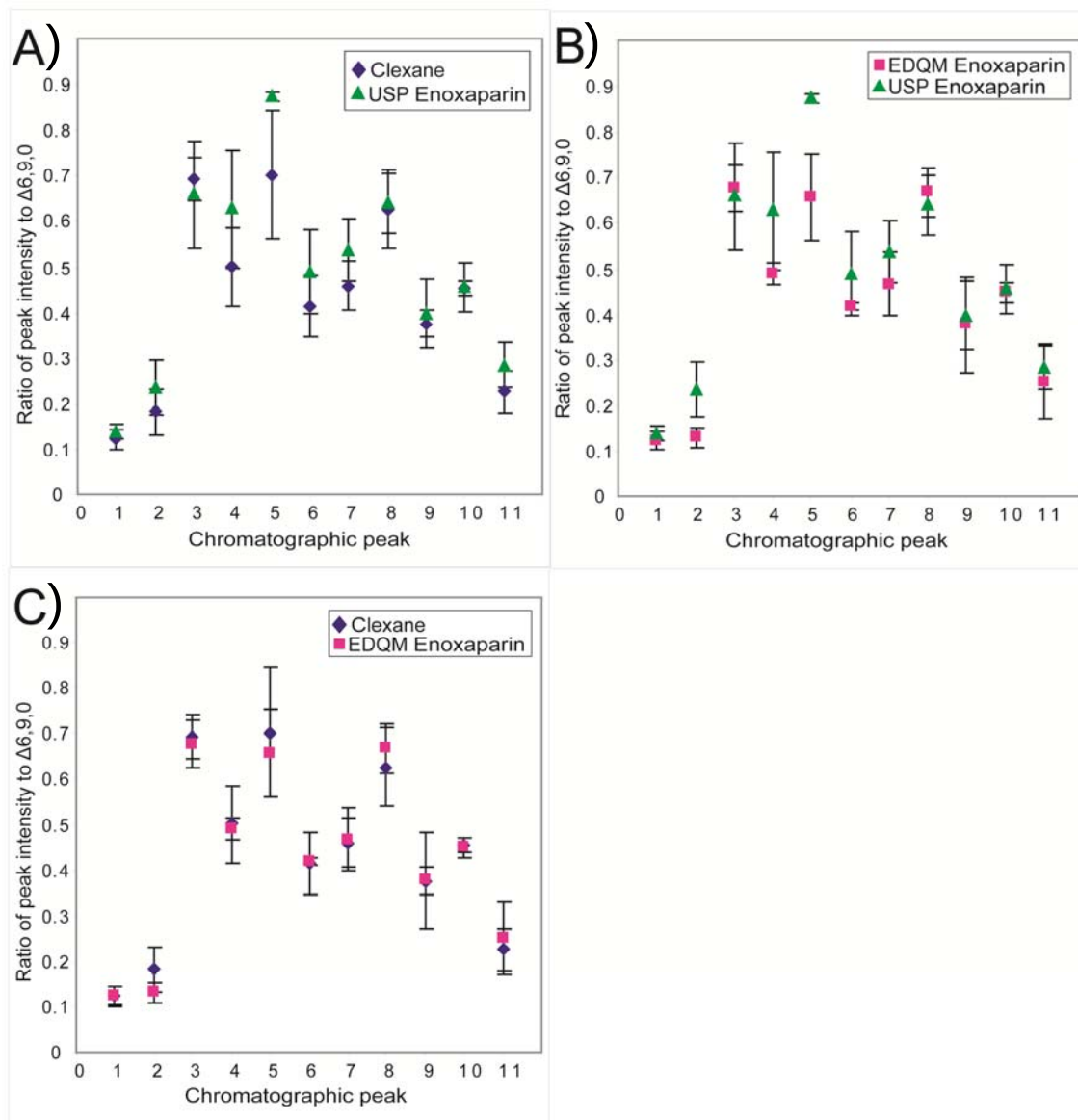


Figure 5.12. Intensities of selected chromatographic peaks as labeled in Figure 5.11. Samples were measured in triplicate and plotted error represents 2 times the standard deviation. Peak intensities are normalized to the most intense peak ($\Delta 6,9,0$).

5.4. Conclusions

In the current study PTA was found to outperform TrBA and DBA as an IPR when used with ACN giving the best resolution over the widest oligomer size range and was thus chosen as the method for LMWH fingerprinting. DBA was shown to give superior isomer resolution for lower molecular weight oligosaccharides when used in conjunction with MeOH, but was not effective in resolving larger oligosaccharides. The method outlined in this manuscript gave reproducible retention times which are important in comparing LMWH preparations and for evaluating lot-to-lot variability. An analysis time of 40 min was found to provide sufficient resolving power for fingerprinting tinzaparin and enoxaparin, but the enhanced resolution offered by longer methods (e.g., 100 min) could be useful for component-level characterization of samples identified as suspect in an initial screen or for the development of novel heparin preparations targeting conditions other than thrombosis. The work presented herein was acquired using a Q-TOF mass spectrometer which provided accurate mass data and allowed extraction of important structural information for the LMWH component oligosaccharides. It should be noted that the mass spectrometer used in this work is an older (circa 2004) model, but the provided mass accuracy is sufficient to perform the attribution of every mass signal. However, better mass signal resolution allowing detection of larger oligosaccharides (dp >22) could likely be achieved using a newer instrument or an alternative mass analyzer such as an FT-MS or orbitrap. The use of different mass detectors does not affect the conclusions drawn about the optimum separation conditions.

The work presented in this chapter was performed to develop analytical methods which are orthogonal to the NMR studies in chapters 2, 3, and 4. The complexity and prevalence of heparin and HS requires a wide array of analytical methods and approaches. Whether the end goal involves tailoring the manufacturing process of a heparin product, ensuring product quality, or characterizing non-standard materials, no single analytical method or instrument is capable of providing all of the pertinent information needed.

This dissertation presents new methods for the analysis of GAGs by both NMR and liquid chromatography. While both approaches can be used to characterize GAGs, they each provide different information. The NMR is more global in nature. The spectra generated represent the composite of all of the individual electronic environments within the active volume of the NMR probe. When a sample is pure, as for an isolated or synthesized oligosaccharide, the ^1H NMR spectrum can be used to derive the absolute structure of that oligosaccharide. It designates exact points of sulfation, identifies the uronic acid epimers and monosaccharide order. When the sample is a mixture, (e.g. LMWH) NMR spectra can provide relative amounts of each type of microenvironment through monosaccharide analysis using the $[^1\text{H}, ^{13}\text{C}]$ HSQC spectrum or by evaluation of the regional microenvironment with the $[^1\text{H}, ^{15}\text{N}]$ HSQC experiment as demonstrated in Chapter 4. This information is independent of average molecular weight or molecular weight distribution of the sample. The data derived from the RPIP-UPCL-MS analysis presented in this chapter provides a complementary view of an LMWH sample. Isolated oligosaccharides can be analyzed for their purity and their parent m/z , but assignment of

sulfation and epimerization are not straight forward and monosaccharide order is difficult to obtain. RPIP-UPLC-MS analysis excels in teasing out individual oligosaccharides in a mixture. It can generate profiles that are unique to individual LMWH preparations and provide information about molecular weight distributions and the relative abundance of individual oligosaccharides. While both NMR and RPIP-UPLC-MS are extremely powerful methods, as demonstrated by the results presented in this dissertation, they are complementary each providing orthogonal information about a sample.

While much of the work presented in this dissertation has been focused on heparin and HS, many of the tools developed are applicable to other polymeric or anionic compounds. The structural and compositional complexity of GAGs provides a worst-case-scenario for the development of analytical methods and truly challenges the capacity of analytical chemistry. We hope our contributions to this field facilitate its overall advancement and aid in the discovery and understanding of complex carbohydrates. With the completion of this body of work we answer many questions while also raising many new ones. In Chapter 6 we will summarize the conclusions drawn from this body of work and discuss what new questions we are now within reach.

5.5 References

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CHAPTER SIX

Conclusions and future directions

6.1 Conclusions

Heparin and heparan sulfate (HS) are members of a class of linear anionic carbohydrate polymers. They are heterogeneous in composition and polydisperse in length. While the structural complexity of this class of biomolecules leads to its diverse array of biological activities, it also complicates its chemical analysis. In this dissertation we have proposed methods for the characterization of heparin and HS using NMR and UPLC-MS platforms.

In Chapter 2, we presented the crux of the NMR research presented herein. This chapter introduces the solution conditions that were found to facilitate the ^1H and ^{15}N NMR detection of the biologically important sulfamate (NHSO_3^-) groups of the *N*-sulfoglucosamine (GlcNS) residues found in heparin and HS. Our studies demonstrated that the ^1H and ^{15}N chemical shifts of the sulfamate group resonances of heparin-derived oligosaccharides are sensitive to the carbohydrate sequence. Using the HSQC-TOCSY experiment, the chemical shifts of carbon-bound protons of the glucosamine ring can even be detected through the well-resolved ^1H and ^{15}N resonances of the sulfamate group. Measurements of mono-, di- and larger oligosaccharides revealed that the pH at which a sulfamate proton exchanges most slowly with water is correlated to the local charge

density, as increasing the anionic charge shifted the pH of the exchange rate minimum to more basic values.

In Chapter 3, the pH- and temperature dependence of the sulfamate ^1H resonances were used to probe additional structural elements that affect the rate of exchange of the sulfamate protons with water. This study identified a persistent intramolecular hydrogen bond between the sulfamate group NH and the adjacent 3-O sulfo group of the central GlcNS(3S)(6S) residue in the synthetic antithrombotic pentasaccharide Arixtra. We postulate that this hydrogen bond contributes to the disproportional contribution of this 3-O sulfo group to the binding affinity between the pentasaccharide and antithrombin III and propose that this hydrogen bond pre-organized the pentasaccharide into a secondary structure that favors binding.

In Chapter 4, a library of heparin- and enoxaparin-derived oligosaccharides, isolated by size-exclusion and strong anion exchange chromatography and characterized using mass spectrometry (MS) and NMR, and chemically modified heparin samples were used to generate a ^1H and ^{15}N chemical shift database of GlcNS residues in various structural environments. This database was used to elucidate the heteronuclear correlations observed in the $[^1\text{H}, ^{15}\text{N}]$ HSQC spectra of unfractionated and low molecular weight heparins (LWMH) as well as commercially available porcine- and human-derived HS. These chemical shift comparisons along with evaluation of the relative solvent exchange rates of the sulfamate group protons enabled the assignments of the major peaks in the $[^1\text{H}, ^{15}\text{N}]$ HSQC spectra of the tested GAG samples. Through this process we identified GlcNS microenvironments corresponding to the reducing and non-reducing

ends, residues adjacent to 2-*O* sulfated and 2-*O* unsulfated uronic acids, residues chemically modified to 1,6 anhydroglucosamine, and most notably the GlcNS(3S)(6S) adjacent to a 2-*O* sulfated uronic acid, a key structural element for high affinity antithrombin binding. This work paves the way for the use of [^1H , ^{15}N] HSQC measurements for quality assurance of pharmaceutical heparin samples and for characterization of other GAG-related compounds, for example intermediates or oligosaccharide products of synthetic or semi-synthetic routes.

Chapter 5 focused on the reversed-phase ion-pairing chromatographic analysis of LMWHs using MS detection. In this chapter we compared methanol and acetonitrile as mobile phases and tributylamine, dibutylamine and pentylamine as ion-pairing reagents (IPRs). The best separation conditions were found using acetonitrile as the mobile phase and pentylamine as the IPR. A separation time of 40 min was found to be sufficient to detect differences in oligosaccharide composition in tinzaparin and enoxaparin. These separation conditions were even able to identify small differences in composition in similar enoxaparin preparations.

6.2 Future directions

The work outlined in this dissertation is focused on developing new analytical tools for the characterization of GAGs. In addition to demonstrating new NMR and LC-MS methods for heparin and HS characterization, the work described in this dissertation also includes new ways to use these tools. In Chapter 3 we discuss the pH-dependence of the exchange rate of sulfamate protons with the bulk solvent. We noticed a correlation

between the degree of negative charge surrounding a sulfamate group and the pH that minimized the exchange rate of the sulfamate protons. This correlation is only touched on in this study and was not fully investigated. One future research direction could be to perform pH titration of a series of saccharides and determine the effect of oligosaccharide size and charge on the pH-dependent exchange rates. If increasing the negative charge really is correlated to the pH-dependent exchange rates, then a systematic shift of the exchange minimized pH further into the basic pH regime should be observed. One could envision the comparison of the fully sulfated di-, tetra- hexa- and octasaccharides isolated from a heparin digest. These should correspond to the most abundant oligosaccharides in each of the size-uniform fractions simplifying isolation. A similar study could be performed using an acetylated series of heparin or HS oligosaccharides. Additionally, we could envision this type of analysis on a series of uniform-sized oligosaccharides which only differ by charge. The results of this study would increase our understanding of the polyelectrolyte character of GAG oligosaccharides and their solution phase physico-chemical behavior.

In Chapter 3 we also probe the ability of sulfamate groups to report on hydrogen bonding. Our measurements of sulfamate proton exchange parameters revealed the presence of the first intramolecular hydrogen bond in a heparin oligosaccharide in aqueous solution. Similar experiments could be used to probe protein-carbohydrate interactions through intermolecular hydrogen bonding. While it has been thought that the anionic charge provides the main contribution to binding to lysine and arginine residues in proteins such as antithrombin III, it may be possible that the sulfamate NH groups

could also interact with uncharged side chains through hydrogen bonding. By producing ^{15}N -enriched HS from cell culture or isotopically-labeled compounds by chemo-enzymatic synthesis, one could envision performing binding experiments to measure chemical shift and proton exchange rate studies using solutions of isolated oligosaccharides with and without the target protein.

The ability to detect sulfamate groups by ^1H NMR can also be applied to probe the contribution of sulfamate groups to binding by using the saturation transfer difference (STD) experiment. Shortly after we published our work describing the detection of sulfamate groups by NMR, Guerrini et al. used these resonances in a STD study of the binding of 3-O sulfated oligosaccharides to antithrombin III.¹ This type of experiment could be extended to other protein targets as saturation transfer is dependant on the proximity of ligand protons to the irradiated protein protons. In such studies the sulfamate proton resonances could be used to report on electrostatic interactions of the sulfamate group that would otherwise go undetected.

In Chapter 4 we elucidate the [^1H , ^{15}N] HSQC spectra of enoxaparin, and intact heparin and HS. In carrying out these studies, we found that the heteronuclear correlation for the sulfamate groups of the rare GlcNS(3S)(6S) residue gave an enhanced and discrete signal compared to the other sulfamate groups. This unique spectral signature could be used in future studies to search for 3-O sulfated residues in more complex mixtures of oligosaccharides. When isolating oligosaccharides from enzymatic digests of LMWHs, after size fractionation by size-exclusion chromatography (SEC), size-uniform fractions are typically pooled prior to charge fractionation by strong anion exchange

chromatography (SAX). Previous lab members have shown that the fractions in size-uniform SEC peaks are not homogenous with respect to the oligosaccharide composition across the SEC peak. For example, using capillary electrophoresis profiling Eldridge et al. demonstrated that the disaccharide composition of SEC fractions containing the early eluting disaccharides differed from the later eluting fractions.² Profiling SEC fractions of larger oligosaccharides using CE is not practical due to the complexity of the samples, and the lack of authentic standards for peak identification. The [¹H, ¹⁵N]HSQC experiment could be used to screen for 3-*O*-sulfo containing fractions across a single SEC peak. These HSQC results could pinpoint the SEC fractions containing oligosaccharides of interest and only these isolated fractions would need to be subjected to further separation by SAX. This should reduce the complexity of the SAX chromatogram, and would require analysis of much less material (i.e. fewer injections), facilitating the isolation of rare 3-*O*-sulfated oligosaccharides for further study.

Another question that the results presented in Chapter 4 raised is the effect of counter ions on the NH exchange rate in mixtures of larger oligosaccharides. In Chapter 4 we focused on minimizing the ionic strength of the solutions to minimize the potential for exchange catalysis. We know that the heparin oligosaccharides act as polyelectrolytes in solution due to their high anionic charge density. Future studies could explore the effect of different types and concentrations of counter ions on the cross peak intensity in the [¹H, ¹⁵N] HSQC spectra. The presence of larger or more highly charged cations in solution could further limit the accessibility of the catalytic hydroxide ions to the sulfamate groups slowing exchange. Also, adding divalent cations such as calcium, which

are known to bind to heparin, may decrease the flexibility of the oligosaccharides which could potentially result in sharper peaks due less averaging of possible solution structures on the NMR time scale.

The work in Chapter 5 made good use of the available instruments for LC-MS profiling of LMWH samples, this approach has the potential to provide even more detailed structural information. Though our Q-TOF mass spectrometer provided excellent mass accuracy, the sensitivity is not ideal for detection of heparin-derived oligosaccharides. If the reversed-phase ion-pairing separation could be performed using an ultraperformance liquid chromatograph connected to Fourier transform mass spectrometer, the results would be even more informative. The additional mass resolution would help tease out coeluting size isomers differing only in their charge. The increased sensitivity would also aid in the detection of very low abundance oligosaccharides. Using a newer electrospray ionization source would also have the potential to significantly increase the range of oligosaccharides which are detectable by increasing the ionization efficiency later in the separation where chromatographic overlap becomes a bigger issue.

GAG analysis and characterization remains a dynamic process; new methods are developed while new challenges arise. The heparin contamination event of 2008 sparked renewed interest in characterizing and quantifying heparin adulterants, process contaminants and naturally occurring minor components. Many new analytical methods were developed in response to this need and new requirements for the chemical characterization of heparin drugs have been adopted by regulatory agencies. The successful approval of generic enoxaparin has brought with it additional challenges for

manufacturers who must demonstrate to regulators that their new product is equivalent, or biosimilar, to a currently approved heparin drug. Given the inherent complexity of LMWHs, this presents a significant analytical challenge for both manufacturers and regulators.

Now we find ourselves at a new frontier, synthetic heparin. To eliminate the uncertainty of raw material quality and potential adverse effects, there is a growing shift towards synthetic heparin mimetics.^{3,4} The first marketed product in this class is the synthetic antithrombotic drug Arixtra, but it is likely that more will follow. The homogeneity of synthetic oligosaccharides has the potential to reduce unintended cross reactivity compared to the broad mixture of oligosaccharides found in unfractionated and LMW heparins. While beneficial, this emerging class of synthetic heparins also presents the need for new and sensitive analytical tools to ensure consumer safety as well as aid in the product development process. As the heparin industry develops and matures, the supporting analytical chemistry will need to continue to do so as well.

6.3 References

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Appendix 1

HSQC-TOCSY-W5 pulse program used in manuscript.

```
;HSQC-TOCSY
;2D H-1/X correlation via double inept transfer
; using sensitivity improvement and DIPSI2
; for homonuclear Hartman-Hahn mixing
;phase sensitive using Echo/Antiecho-TPPI gradient selection
;with decoupling during acquisition and W5 Watergate solvent suppression.
;
;$CLASS=HighRes
;$DIM=2D
;$TYPE=
;$SUBTYPE=
;$COMMENT=
```

```
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
```

```
"p2=p1*2"
"p4=p3*2"
"d0=3u"
"d11=30m"
"d13=4u"
"d4=1s/(cnst2*4)"
```

```
# ifdef LABEL_CN
"p22=p21*2"
# else
# endif /*LABEL_CN*/
```

```
"FACTOR1=(d9/(p6*115.112))/2+0.5"
"l1=FACTOR1*2"
```

```
"DELTA1=d13+p16+d16+4u"
```

```
# ifdef LABEL_CN
"DELTA=p16+d16+larger(p2,p22)+d0*2"
# else
"DELTA=p16+d16+p2+d0*2"
# endif /*LABEL_CN*/
```

```
1 ze
```

```
# ifdef LABEL_CN
d11 pl12:f2 pl16:f3
```

```

2 d1 do:f2 do:f3
  10u pl2:f2 pl3:f3
# else
  d11 pl12:f2
2 d1 do:f2
  10u pl2:f2
# endif /*LABEL_CN*/

3 (p1 ph1)
  d4
  (center (p2 ph1) (p4 ph6):f2 )
  d4 UNBLKGRAD
  p28 ph1
  d13
  (p1 ph2)
  5u
  p16:gp3
  d16
  (p3 ph3):f2
  d0

# ifdef LABEL_CN
  (center (p2 ph7) (p22 ph1):f3 )
# else
  (p2 ph7)
# endif /*LABEL_CN*/

  d0
  p16:gp1*EA
  d16
  (p4 ph4):f2
  DELTA
  (center (p1 ph1) (p3 ph4):f2 )
  d24
  (center (p2 ph1) (p4 ph1):f2 )
  d24
  (center (p1 ph2) (p3 ph5):f2 )
  d4
  (center (p2 ph1) (p4 ph1):f2 )
  d4 pl10:f1

4 p6*3.556 ph22
  p6*4.556 ph24
  p6*3.222 ph22
  p6*3.167 ph24
  p6*0.333 ph22
  p6*2.722 ph24
  p6*4.167 ph22
  p6*2.944 ph24
  p6*4.111 ph22

  p6*3.556 ph24

```

;begin DIPSI2

p6*4.556 ph22
p6*3.222 ph24
p6*3.167 ph22
p6*0.333 ph24
p6*2.722 ph22
p6*4.167 ph24
p6*2.944 ph22
p6*4.111 ph24

p6*3.556 ph24
p6*4.556 ph22
p6*3.222 ph24
p6*3.167 ph22
p6*0.333 ph24
p6*2.722 ph22
p6*4.167 ph24
p6*2.944 ph22
p6*4.111 ph24

p6*3.556 ph22
p6*4.556 ph24
p6*3.222 ph22
p6*3.167 ph24
p6*0.333 ph22
p6*2.722 ph24
p6*4.167 ph22
p6*2.944 ph24
p6*4.111 ph22
lo to 4 times l1

;end DIPSI2

4u pl1:f1
(p1 ph1)
DELTA1
(p2 ph1)
d13
p16:gp2

;start watergate

50u pl5:f1 UNBLKGRAD
p16:gp4
d16
p27*0.087 ph14
d19*2
p27*0.206 ph14
d19*2
p27*0.413 ph14
d19*2
p27*0.778 ph14
d19*2
p27*1.491 ph14
d19*2
p27*1.491 ph15
d19*2

```

p27*0.778 ph15
d19*2
p27*0.413 ph15
d19*2
p27*0.206 ph15
d19*2
p27*0.087 ph15
50u
p16:gp4
d16

4u

p16:gp5
d16
p27*0.087 ph14
d19*2
p27*0.206 ph14
d19*2
p27*0.413 ph14
d19*2
p27*0.778 ph14
d19*2
p27*1.491 ph14
d19*2
p27*1.491 ph15
d19*2
p27*0.778 ph15
d19*2
p27*0.413 ph15
d19*2
p27*0.206 ph15
d19*2
p27*0.087 ph15
p16:gp5
d16

;end watergate

# ifdef LABEL_CN
d16 pl12:f2 pl16:f3
4u BLKGRAD
go=2 ph31 cpd2:f2 cpd3:f3
d1 do:f2 do:f3 mc #0 to 2
# else
d16 pl12:f2
4u BLKGRAD
go=2 ph31 cpd2:f2
d1 do:f2 mc #0 to 2
# endif /*LABEL_CN*/

F1EA(igrad EA & ip5*2, id0 & ip3*2 & ip6*2 & ip31*2)
exit

```

```

ph1=0
ph2=1
ph3=0 2
ph4=0 0 2 2
ph5=1 1 3 3
ph6=0
ph7=0 0 2 2
ph14=0
ph15=2
ph22=3
ph24=1
ph31=0 2 2 0

```

```

;pl1 : f1 channel - power level for pulse (default)
;pl2 : f2 channel - power level for pulse (default)
;pl3 : f3 channel - power level for pulse (default)
;pl5 : f1 watergate power [same as proton]
;pl10: f1 channel - power level for TOCSY-spinlock
;pl12: f2 channel - power level for CPD/BB decoupling
;pl16: f3 channel - power level for CPD/BB decoupling
;p1 : f1 channel - 90 degree high power pulse
;p2 : f1 channel - 180 degree high power pulse
;p3 : f2 channel - 90 degree high power pulse
;p4 : f2 channel - 180 degree high power pulse
;p6 : f1 channel - 90 degree low power pulse
;p16: homospoil/gradient pulse [1 msec]
;p22: f3 channel - 180 degree high power pulse
;p27: watergate pulse [same as proton]
;p28: f1 channel - trim pulse [1 msec]
;d0 : incremented delay (2D) [3 usec]
;d1 : relaxation delay; 1-5 * T1
;d4 : 1/(4J(XH))
;d9 : TOCSY mixing time
;d11: delay for disk I/O [30 msec]
;d13: short delay [4 usec]
;d16: delay for homospoil/gradient recovery
;d19: watergate delay
;d24: 1/(8J)XH for all multiplicities
; 1/(4J)XH for XH
;cnst2: = J(XH)
;l1: loop for DIPSI cycle: ((p6*115.112) * l1) = mixing time
;in0: 1/(2 * SW(X)) = DW(X)
;nd0: 2
;NS: 1 * n
;DS: >= 16
;td1: number of experiments
;FnMODE: echo-antiecho
;cpd2: decoupling according to sequence defined by cpdprg2
;cpd3: decoupling according to sequence defined by cpdprg3
;pcpd2: f2 channel - 90 degree pulse for decoupling sequence
;pcpd3: f3 channel - 90 degree pulse for decoupling sequence

```

```

;use gradient ratio:      gp 1 : gp 2
;                        80 : 20.1   for C-13
;                        80 : 8.1    for N-15

;for z-only gradients:
;gpz1: 80%
;gpz2: 20.1% for C-13, 8.1% for N-15
;gpz3: -45%
;gpz4: 34%
;gpz5: 22%

;use gradient files:
;gpnam1: SMSQ10.100
;gpnam2: SMSQ10.100

                                ;preprocessor-flags-start
;LABEL_CN: for C-13 and N-15 labeled samples start experiment with
;      option -DLABEL_CN (eda: ZGOPTNS)
                                ;preprocessor-flags-end

;$Id: hsqcdietgps,v 1.3.8.1 2005/11/10 13:18:57 ber Exp $

```

Appendix 2

Example fitting of NMR spectral data for GlcNS

tools and define fitting functions

```
<< "NonlinearRegression`";

gensim[datapairs_, params_] :=
  Module[{table, functiontable, paramtable, model, x, simulation},

    table = If[Dimensions[params[[1]]] == {}, {params}, params];
    functiontable = Table[table[[i, 1]], {i, Length[table]};
    paramtable = Table[Drop[table[[i]], 1], {i, Length[table]};
    model = Sum[Apply[functiontable[[i]], Append[paramtable[[i, j]], x]],
      {i, Length[paramtable]}, {j, Length[paramtable[[i]]}] // N;
    simulation = Table[{datapairs[[i, 1]], model /. x -> datapairs[[i, 1]]},
      {i, Length[datapairs]}]
  ]

fitdatatight[datapairs_, startparams_] :=
  (*
    This function fits data asking for tighter accuracy
  *)

  Module[{startparamtable, functiontable, paramtable, paramnames, model,
    startparampairs, fitsubs, resulttable},

    startparamtable = If[Dimensions[startparams[[1]]] == {}, {startparams},
      startparams];
    functiontable = Table[startparamtable[[i, 1]],
      {i, Length[startparamtable]};
    paramtable = Table[Drop[startparamtable[[i]], 1],
      {i, Length[startparamtable]};
    paramnames = Table[ToExpression["a$$" <> ToString[i] <> ToString[j] <>
      ToString[k]], {i, Length[paramtable]}, {j, Length[paramtable[[i]]},
      {k, Length[paramtable[[i, j]]}];
    model = Sum[Apply[functiontable[[i]], Append[paramnames[[i, j]], x]],
      {i, Length[paramnames]}, {j, Length[paramnames[[i]]}];
    startparampairs = MapThread[{#1, #2} &, {Flatten[paramnames],
      Flatten[paramtable]};
    fitsubs = NonlinearRegress[datapairs, model, startparampairs, x,
      ShowProgress -> False, AccuracyGoal -> 10, PrecisionGoal -> 10,
      RegressionReport -> BestFitParameters];
    resulttable = Table[Prepend[paramnames[[i]], functiontable[[i]]],
```

```

    {i, Length[functiontable]}} /. fitsubs[[1, 2]];
Return[If[Dimensions[startparams][[1]] == {}, resulttable[[1]],
resulttable]]
]
fitdataerr[datapairs_, startparams_] :=
(* This function fits a set of data to an arbitrary number of functions
which have been predefined by the user (such as the functions gaussian
and lorentzian defined above). The data set must be specified in the
form of a list of data pairs. The predefined functions must be analytic
functions of the form
        function[a1_,a2_,a3_,...,x_]
where (a1,a2,a3,...) are the parameters to be fit and x is the
independent variable (and appears last in the argument list).

To fit a data set to one or more instances of the same functional
form (for instance, to fit a spectrum to one or more Gaussians at once),
the argument "startparams" should be specified in the form:

        {function,{a11,a12,a13,...},{a21,a22,a23,...},...}

To combine different functional forms in the same fit, with possibly
multiple instances of the each functional form (such as a fit to
several Gaussians as well as several Lorentzians at once) the argument
"startparams" should be specified in the form:

        {{function1,{a111,a112,a113,...},{a121,a122,a123,...},...},
        {function2,{a211,a212,a213,...},{a221,a222,a223,...},...},
        ...
        {functionN,{aN11,aN12,aN13,...},{aN21,aN22,aN23,...},...}}
*)

Module[{startparamtable, functiontable, paramtable, paramnames, model,
startparampairs, fitsubs, resulttable},

startparamtable = If[Dimensions[startparams][[1]] == {}, {startparams},
startparams];
functiontable = Table[startparamtable[[i, 1]],
{i, Length[startparamtable]}};
paramtable = Table[Drop[startparamtable[[i]], 1],
{i, Length[startparamtable]}};
paramnames = Table[ToExpression["a$$" <> ToString[i] <> ToString[j] <>
ToString[k]], {i, Length[paramtable]}, {j, Length[paramtable[[i]]}],
{k, Length[paramtable[[i, j]]]}};

```

■ Directories & settings

```
In[1]:= DATA = "C:\\Users\\Derek\\Desktop\\Nitrogen kinetics\\FDPX new temp\\";
```

```

model = Sum[Apply[functiontable[[i]], Append[paramnames[[i, j]], x]],
  {i, Length[paramnames]}, {j, Length[paramnames[[i]]}];
startparampairs = MapThread[{#1, #2} &, {Flatten[paramnames],
  Flatten[paramtable]};
fitsubs = NonlinearRegress[datapairs, model, startparampairs, x,
  ShowProgress -> False, AccuracyGoal -> 3, PrecisionGoal -> 3,
  RegressionReport -> SummaryReport];
Print[fitsubs];
resulttable = Table[Prepend[paramnames[[i]], functiontable[[i]]],
  {i, Length[functiontable]} /. fitsubs[[1, 2]];
Return[If[Dimensions[startparams[[1]]] == {}, resulttable[[1]],
  resulttable]]
]

```

```

lorentzian[position_, intensity_, width_, x_] :=
  intensity / width / (1 + 4 ((x - position) / width)^2)
rlorentzian[position_, intensity_, width_, phase_, x_] :=

$$\frac{\text{intensity} \left( \frac{\cos[\text{phase}]}{\text{width}} - \frac{\sin[\text{phase}]^2 (x - \text{position})}{\text{width}^2} \right)}{1 + 4 \left( \frac{x - \text{position}}{\text{width}} \right)^2}$$


```

```

lorentzian1[position_, intensity_, width_, x_] :=
  lorentzian[position + 11.2 / 2, intensity / 2, width, x] +
  lorentzian[position - 11.2 / 2, intensity / 2, width, x]
lorentzian3[position_, intensity_, width_, x_] :=
  lorentzian[position + 8.4 / 2, intensity / 2, width, x] +
  lorentzian[position - 8.4 / 2, intensity / 2, width, x]
lorentzian5[position_, intensity_, width_, x_] :=
  lorentzian[position + 8.4 / 2, intensity / 2, width, x] +
  lorentzian[position - 8.4 / 2, intensity / 2, width, x]
lorentzianJ[position_, intensity_, width_, J_, x_] :=
  lorentzian[position + J / 2, intensity / 2, width, x] +
  lorentzian[position - J / 2, intensity / 2, width, x]
rlorentzianJ[position_, intensity_, width_, J_, ph_, x_] :=
  rlorentzian[position + J / 2, intensity / 2, width, ph, x] +
  rlorentzian[position - J / 2, intensity / 2, width, ph, x]
line[b_, m_, x_] := m x + b

```

General::obspkg :

NonlinearRegression` is now obsolete. The legacy version being loaded may conflict with current Mathematica functionality. See the Compatibility Guide for updating information. >>

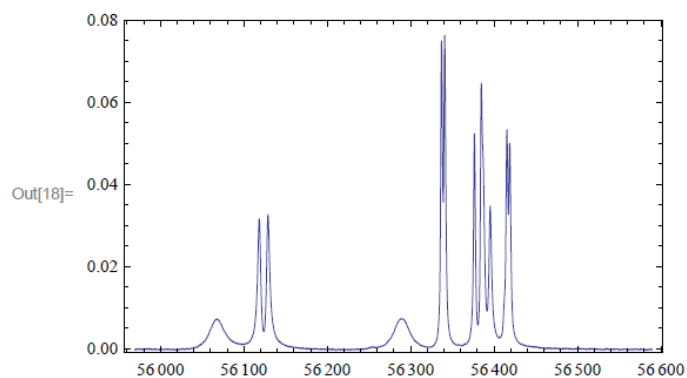
```

eadjcamp[filename_, pointstartin_, pointendin_] :=
Module[{datalist}, findstring = "$$ End of Bruker specific parameters";
  datafile = OpenRead[filename];
  While[Read[datafile, Record] # findstring];
  Read[datafile, Record];
  Read[datafile, Record];
  Read[datafile, Record];
  Read[datafile, Word];
  xfactor = Read[datafile, Number];
  Read[datafile, Record];
  Read[datafile, Word];
  firstx = Read[datafile, Number];
  Read[datafile, Record];
  Read[datafile, Record];
  Read[datafile, Word];
  maxy = Read[datafile, Number];
  maxy = 108;
  Read[datafile, Record];
  Read[datafile, Word];
  npoints = Read[datafile, Number];
  Read[datafile, Record];
  Read[datafile, Record];
  linestart = Floor[pointstartin / 4 + 3 / 4];
  lineend = Floor[pointendin / 4 + 3 / 4];
  pointstart = 4 * linestart - 3;
  pointend = 4 * lineend;
  refstart = -firstx + xfactor (pointstart - 1) + 60 000;
  Do[Read[datafile, Record], {i, 1, lineend - 1}];
  temp = Table[Table[Read[datafile, {Number, Number, Number, Number, Number}],
    {j, linestart, lineend}];
  datalist = {};
  Do[datalist =
    Join[datalist, Table[{refstart + (4 (i - 1) + j - 2) * xfactor, temp[[i, j]] / maxy} // N,
      {j, 2, 5}], {i, 1, lineend - linestart + 1}];
  Close[filename];
  datalist]

```

■ Fit a spectrum to extract linewidths

```
In[15]:= k = 1024;
file = "FDPX_275.32.dx";
datapairs = readjcamp[DATA <> file, 25 k, 31 k];
ps1 = ListPlot[ datapairs, Joined → True, PlotRange → {- .001, 0.08}, Frame → True]
```



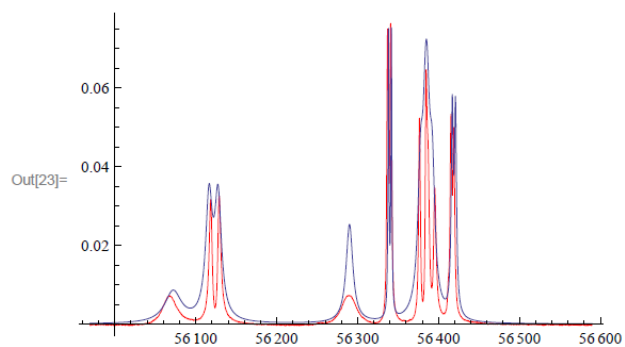
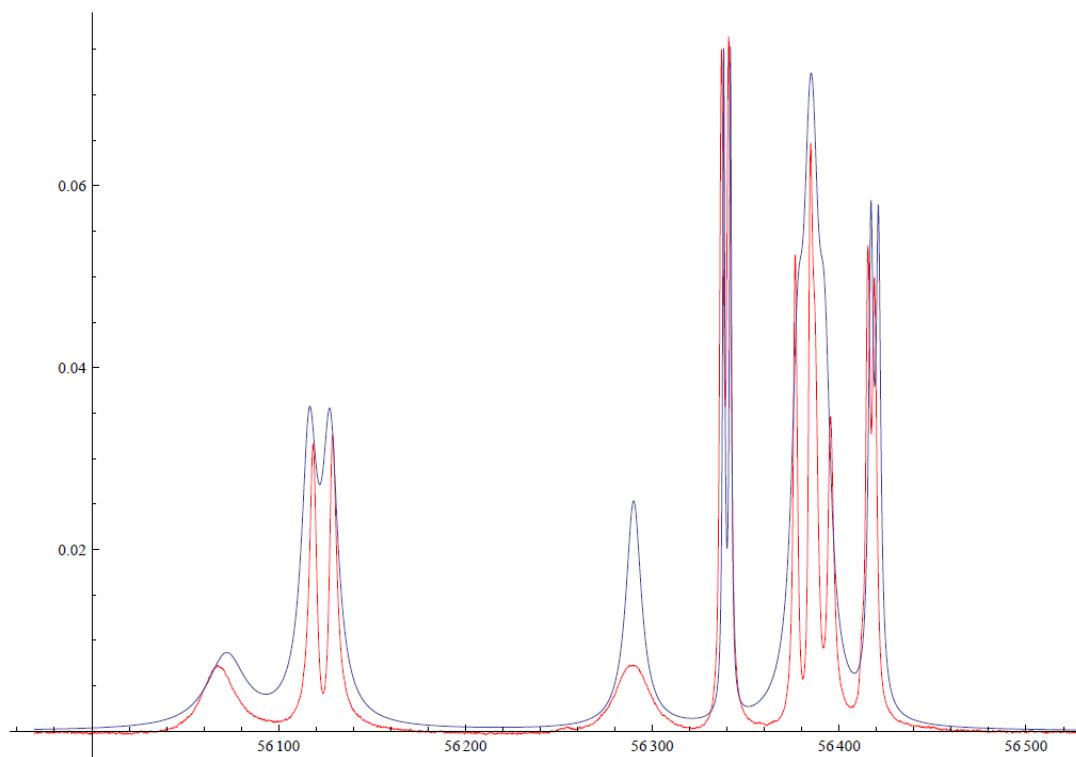
```
In[19]:= startparams = {{rlorentzian, {56 072, 0.2, 25, 0}},
    {lorentzian1, {56 122, 0.6, 10}},
    {lorentzianJ, {56 340, 0.2, 1.4, 3.6}},
    {rlorentzian, {56 290, 0.25, 10, 0}},
    {lorentzian3, {56 382, 0.5, 8}},
    {lorentzian5, {56 388, 0.5, 8}},
    {lorentzianJ, {56 419, 0.3, 3, 4}},
    {line, {0, 0}}}
```

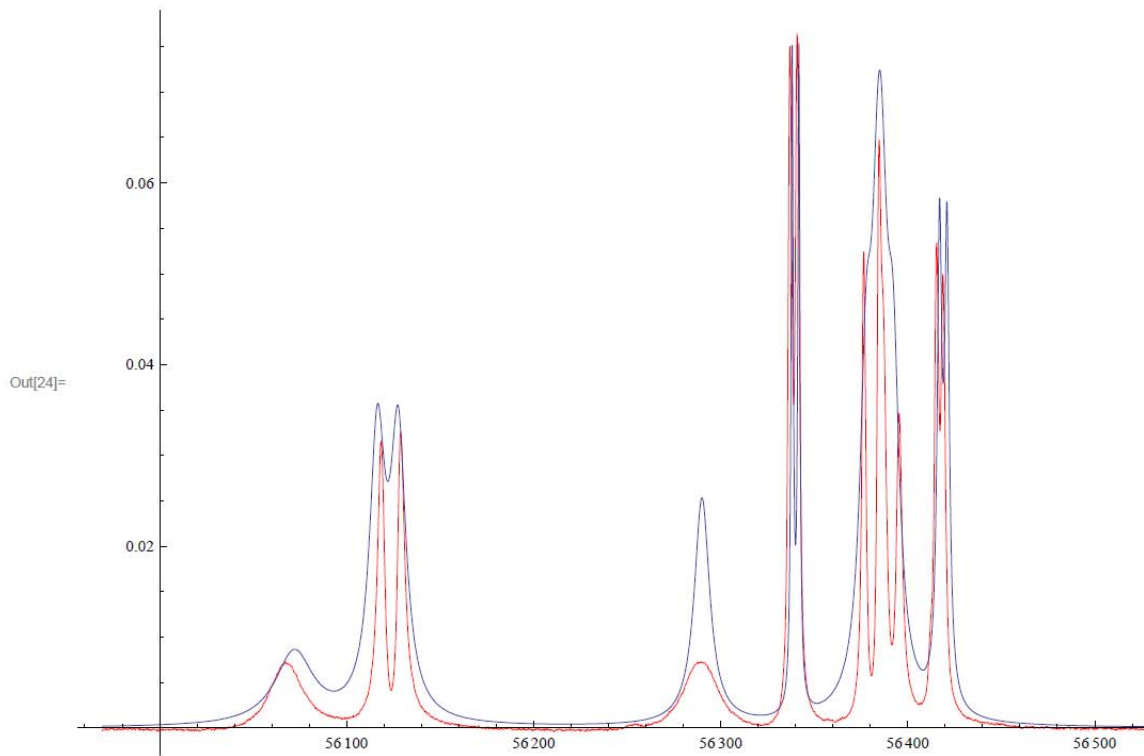
```
Out[19]= {{rlorentzian, {56 072, 0.2, 25, 0}}, {lorentzian1, {56 122, 0.6, 10}},
    {lorentzianJ, {56 340, 0.2, 1.4, 3.6}}, {rlorentzian, {56 290, 0.25, 10, 0}},
    {lorentzian3, {56 382, 0.5, 8}}, {lorentzian5, {56 388, 0.5, 8}},
    {lorentzianJ, {56 419, 0.3, 3, 4}}, {line, {0, 0}}}
```

```

In[20]:= data$sim = gensim[datapairs, startparams];
plot$sim = ListPlot[data$sim, PlotRange → All, Joined → True, DisplayFunction → Identity];
plot$data = ListPlot[datapairs, PlotRange → All,
  Joined → True, DisplayFunction → Identity, PlotStyle → Red];
Show[plot$data, plot$sim, DisplayFunction → $DisplayFunction]

```





```
In[25]= fitresults27532 = fitresults = fitdatatight[datapairs, startparams]
{{rlorentzian,
 {56067.15865709035`, 0.1547317862970949`, 20.47140699276394`, -0.09401607782400405`}},
 {lorentzian1, {56123.77517250944`, 0.26019408830140794`, 4.156536182449785`}},
 {lorentzianJ, {56339.03175088593`, 0.33327798823882737`,
 2.311105811004059`, 3.891275910454362`}}, {rlorentzian,
 {56290.50204751875`, 0.14391066355570364`, 18.048670325491695`, 0.12963997605691668`}},
 {lorentzian3, {56380.612544566866`, -0.23732810932254586`, -2.2186283923780565`}},
 {lorentzian5, {56391.34439842291`, 0.2254010173700532`, 3.313962760959959`}}, {lorentzianJ,
 {56417.14240101527`, 0.24991713280027747`, 2.7247282432299484`, 3.561231202530959`}},
 {line, {-0.026370586378342838`, 4.6258618235233064`*^-7}}}
```

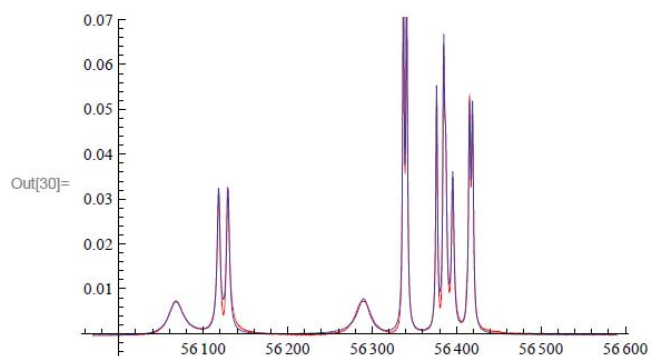
```
Out[25]= {{rlorentzian, {56067.2, 0.154732, 20.4714, -0.0940161}},
 {lorentzian1, {56123.8, 0.260194, 4.15654}},
 {lorentzianJ, {56339., 0.333278, 2.31111, 3.89128}},
 {rlorentzian, {56290.5, 0.143911, 18.0487, 0.12964}},
 {lorentzian3, {56380.6, -0.237328, -2.21863}}, {lorentzian5, {56391.3, 0.225401, 3.31396}},
 {lorentzianJ, {56417.1, 0.249917, 2.72473, 3.56123}}, {line, {-0.0263706, 4.62586 × 10-7}}}
```

```
Out[26]= {{rlorentzian, {56067.2, 0.154732, 20.4714, -0.0940161}},
 {lorentzian1, {56123.8, 0.260194, 4.15654}},
 {lorentzianJ, {56339., 0.333278, 2.31111, 3.89128}},
 {rlorentzian, {56290.5, 0.143911, 18.0487, 0.12964}},
 {lorentzian3, {56380.6, -0.237328, -2.21863}}, {lorentzian5, {56391.3, 0.225401, 3.31396}},
 {lorentzianJ, {56417.1, 0.249917, 2.72473, 3.56123}}, {line, {-0.0263706, 4.62586 × 10-7}}}
```

```

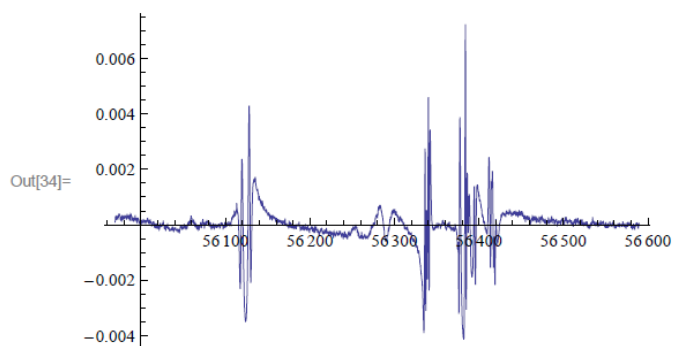
In[27]:= data$sim = gensim[datapairs, fitresults];
plot$sim = ListPlot[data$sim, PlotRange → All, Joined → True, DisplayFunction → Identity];
plot$data = ListPlot[datapairs, PlotRange → {-.005, .07},
    Joined → True, DisplayFunction → Identity, PlotStyle → Red];
Show[plot$data, plot$sim, DisplayFunction → $DisplayFunction]

```



In[31]=

```
In[32]:=
data$res = Table[{datapairs[[i, 1]], datapairs[[i, 2]] - data$sim[[i, 2]]}, {i, Length[datapairs]}
plot$res = ListPlot[data$res, PlotRange -> All, Joined -> True, DisplayFunction -> Identity]
Show[plot$res, DisplayFunction -> $DisplayFunction]
```



■ From linewidths (Alist) vs temperature (Tlist), fit for Activation Energy

```
In[35]:= Tlist = {273.08,
275.32,
277.4,
279.64,
283.84,
286.09,
288.29,
290.17,
291.88,
294.29,
296.41,
298.29,
300.5,
302.38,
306.8,
308.91,
310.74,
312.67,
314.28}
```

```
Out[35]= {273.08, 275.32, 277.4, 279.64, 283.84, 286.09, 288.29, 290.17, 291.88,
294.29, 296.41, 298.29, 300.5, 302.38, 306.8, 308.91, 310.74, 312.67, 314.28}
```

```

In[36]:= Alist = {5.33544,
5.74255,
5.6494,
5.81514,
6.65269,
7.44098,
8.12663,
9.0259,
9.0128,
10.4076,
11.6575,
12.4599,
14.4502,
15.1382,
17.7553,
17.8918,
19.9977,
22.1556,
23.9984}

Out[36]= {5.33544, 5.74255, 5.6494, 5.81514, 6.65269, 7.44098, 8.12663, 9.0259, 9.0128, 10.4076,
11.6575, 12.4599, 14.4502, 15.1382, 17.7553, 17.8918, 19.9977, 22.1556, 23.9984}

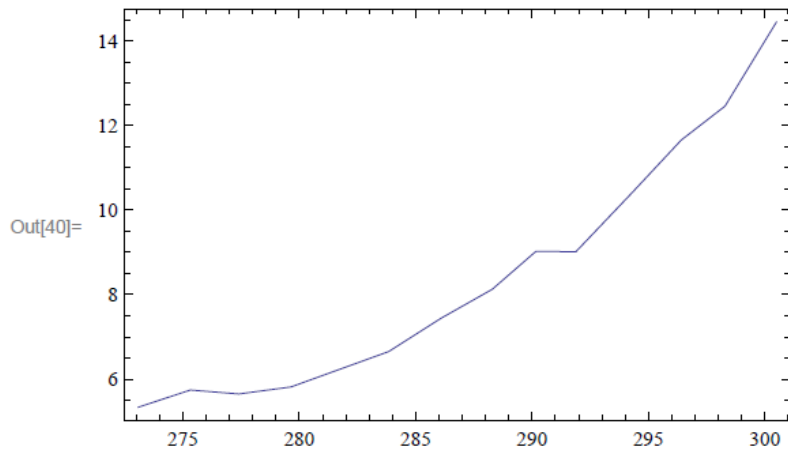
In[37]:= fitA = Table[{Tlist[[i]], Alist[[i]]}, {i, Length[Alist] - 6}]

Out[37]= {{273.08, 5.33544}, {275.32, 5.74255}, {277.4, 5.6494}, {279.64, 5.81514}, {283.84, 6.65269},
{286.09, 7.44098}, {288.29, 8.12663}, {290.17, 9.0259}, {291.88, 9.0128},
{294.29, 10.4076}, {296.41, 11.6575}, {298.29, 12.4599}, {300.5, 14.4502}}

In[38]:= ListPlot[fitA]

```

```
In[39]:= datapairs = fitA;
ps1 = ListPlot[ datapairs, Joined → True, PlotRange → All, Frame → True]
```



```
In[41]:= kb = 1.38 × 10-23
h = 6.626 × 10-34
R = 1.9872
```

Out[41]= 1.38×10^{-23}

Out[42]= 6.626×10^{-34}

Out[43]= 1.9872

```
In[44]:= rate[dg_, T_] := 1 / π kb / h T Exp[- dg / R / T]
```

```
In[45]:= const[a_, T_] := a
```

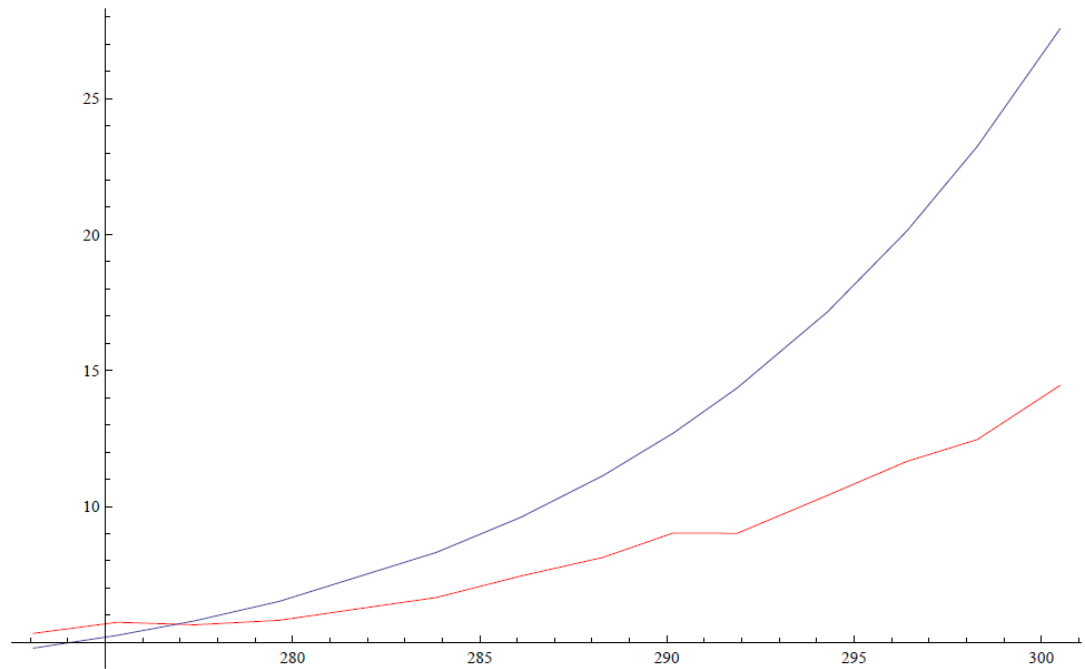
```
In[46]:= startparams = {{rate, {15 000}}, {const, {3}}}
```

Out[46]= {{rate, {15 000}}, {const, {3}}}

In[47]:=

```
data$sim = gensim[datapairs, startparams];  
plot$sim =  
  ListPlot[data$sim, PlotRange → All, Joined → True, DisplayFunction → Identity];  
plot$data = ListPlot[datapairs, PlotRange → All, Joined → True,  
  DisplayFunction → Identity, PlotStyle → Red];  
Show[plot$data, plot$sim, DisplayFunction → $DisplayFunction]
```

Out[50]=



In[51]:= **fitresults = fitdatatight[datapairs, startparams]**

Out[51]= {{rate, {15 553.4}}, {const, {4.80415}}}

```

In[52]:= fitdataerr[datapairs, fitresults]

{BestFitParameters → {a$$111 → 15 553.4, a$$211 → 4.80415},
ParameterCITable → 

|          | Estimate | Asymptotic SE | CI                   |
|----------|----------|---------------|----------------------|
| a\$\$111 | 15 553.4 | 14.4692       | {15 521.6, 15 585.3} |
| a\$\$211 | 4.80415  | 0.115919      | {4.54902, 5.05929}   |

,
EstimatedVariance → 0.0592954, ANOVATable → 

|                   | Model | DF | SumOfSq | MeanSq    |
|-------------------|-------|----|---------|-----------|
| Error             |       | 2  | 1063.16 | 531.581   |
| Uncorrected Total |       | 11 | 0.65225 | 0.0592954 |
| Corrected Total   |       | 13 | 1063.81 |           |
|                   |       | 12 | 102.734 |           |

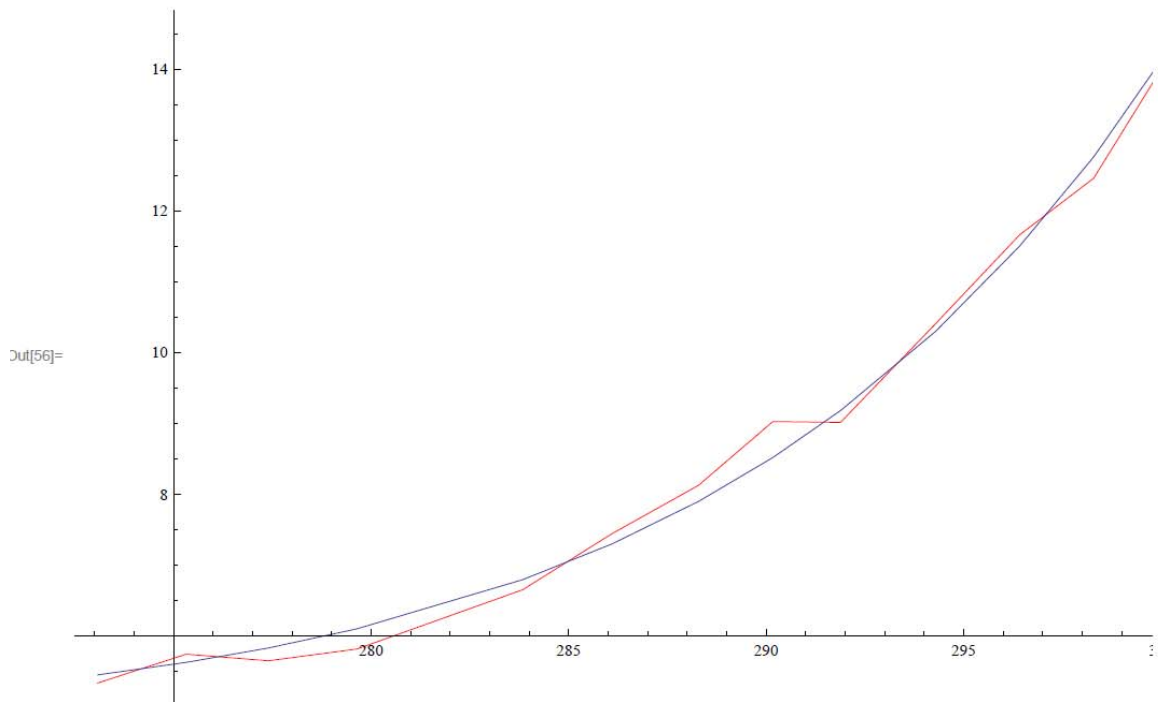
,
AsymptoticCorrelationMatrix →  $\begin{pmatrix} 1. & 0.812747 \\ 0.812747 & 1. \end{pmatrix}$ ,
FitCurvatureTable → 

|                         | Curvature  |
|-------------------------|------------|
| Max Intrinsic           | 0.00040544 |
| Max Parameter-Effects   | 0.0596759  |
| 95. % Confidence Region | 0.50111    |


Out[52]:= {{rate, {15 553.4}}, {const, {4.80415}}}}

In[53]:= data$sim = gensim[datapairs, fitresults];
plot$sim =
  ListPlot[data$sim, PlotRange → All, Joined → True, DisplayFunction → Identity];
plot$data = ListPlot[datapairs, PlotRange → All, Joined → True,
  DisplayFunction → Identity, PlotStyle → Red];
Show[plot$data, plot$sim, DisplayFunction → $DisplayFunction]

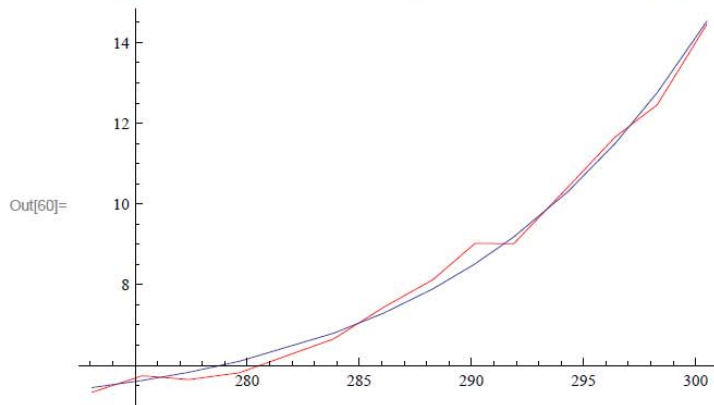
```



```

In[57]:= data$sim = gensim[datapairs, fitresults];
plot$sim =
  ListPlot[data$sim, PlotRange → All, Joined → True, DisplayFunction → Identity];
plot$data = ListPlot[datapairs, PlotRange → All, Joined → True,
  DisplayFunction → Identity, PlotStyle → Red];
Show[plot$data, plot$sim, DisplayFunction → $DisplayFunction]

```



```

In[61]:= data$res =
  Table[{datapairs[[1, 1]], datapairs[[1, 2]] - data$sim[[1, 2]]}, {1, Length[datapairs]}];
plot$res = ListPlot[data$res, PlotRange → All, Joined → True, DisplayFunction → Identity];
Show[plot$res, DisplayFunction → $DisplayFunction]

```

