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Reverse-phase Ion-Pairing Ultra Performance Liquid Chromatography-Mass Spectrometry In Characterization And Fingerprinting Of Diverse Sulfated Glycosaminoglycan Mimetics

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Reverse-phase Ion-Pairing Ultra Performance Liquid Chromatography-Mass Spectrometry In Characterization And Fingerprinting Of Diverse Sulfated Glycosaminoglycan Mimetics

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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May 2013
Dedicated to my parents and my sister

*Your endless love made me the person that I am today.*

*You are the light of my life.*
ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude towards my family, without whose unconditional love and support, I would not be here, on my way to fulfilling my dreams. I would also like to thank my advisor, Dr. Desai, for giving me, a novice, the opportunity to work on the lab’s coveted mass spectrometer! Your timely advice and suggestions have always lent a boost in the quality and quantity of results generated. Thank you for being patient with me for all these years and constantly guiding me on the nuances of writing and presenting scientific literature. I promise that I will get better at it with each day.

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Abstract

REVERSE-PHASE ION-PAIRING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY IN CHARACTERIZATION AND FINGERPRINTING OF DIVERSE SULFATED GLYCOSAMINOGLYCAN MIMETICS

POOJA PONNU SAMY M. S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2013

Director: Dr. UMESH R. DESAI

Professor, Department of Medicinal Chemistry

Heparin is a highly sulfated glycosaminoglycan with potent anticoagulant, antimetastatic, and anti-inflammatory effects. Polymeric and polyanionic nature of heparin makes dosing and side effects a nightmare for healthcare professionals.

Our laboratory has proposed appropriately designed, small, highly sulfated aromatic molecules as potential mimetics of heparin. These easier-to-synthesize small molecules have been shown to possess interesting pharmacological and improved toxicological profiles. However, the detection and characterization of these highly sulfated molecules is challenging.

A robust RP-IP UPLC-MS method was developed to successfully retain, resolve and quantify sulfated non-saccharide GAG mimetics without the requirement of pre- or
post-column derivatization. Comparative analysis reveals intricate dependence of resolution and ionization on the structure of ion-pairing agents. This is the first report showing systematic use of MS cone voltage to fingerprint sulfated GAG mimetics, perhaps eliminating the need for tandem MS techniques.
CHAPTER 1

Introduction

Glycosaminoglycans (GAGs) are a family of linear polysaccharides defined by the presence of acidic groups on repeating disaccharide units and found commonly associated with cell-surface proteins in mammals.\(^1,2\) Examples of GAGs obtained from nature include heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparin and others.\(^3\) GAGs are found in different parts of the human body such as skin, cornea, cartilage, basement membrane etc., and exhibit important roles in cellular growth, differentiation, cell death, regulation of coagulation etc.\(^1,2,4,5\) Much remains to be understood on how they modulate protein function, and this aspect is gaining prominence in current studies. Experimental studies on identifying GAG sequences are being increasingly approached in order to better understand the structural factors affecting their localization in the body and the diversity in their functions.

Heparan sulfate

Heparan sulfate (HS) is a linear, variably sulfated polysaccharide chain composed of uronic acids alternating with glucosamine units.\(^2,3,6\) Uronic acids are sugars with C-6 hydroxyl group oxidized to a carboxylic acid and can exist in HS as either glucuronic acid (uronic acid of glucose) or iduronic acid (C-5 epimer of glucuronic acid). Glucosamines, on the other hand, are sugars with C-2 hydroxyl group replaced by an amine that could be either acetylated to form acetylglucosamine or sulfated to form glucosamine sulfate, both of which are present in HS (see Figure 1.1).
HS is actually a proteoglycan (PG), found ubiquitously on cell-surfaces, and two of which, glypicans and syndecans, have been studied significantly. HSPGs are involved in diverse processes, some of them being recognition and binding extra-cellular matrix to epithelial and endothelial cells; facilitating signaling through activation of seven pass transmembrane receptors and tyrosine kinase receptors; providing as a base for localization of pathogens to adhere, internalize and replicate in host cells etc.

Other GAGs like chondroitin sulfate form the “glycan” part of aggrecans, constituting a major component of cartilage, therefore essential in the treatment of cartilage disorders such as osteoarthritis. Dermatan sulfate is a GAG predominantly found in mammalian skin, associated with glycoproteins such as fibronectin and heparin co-factor II, and is found to be involved in biologically-relevant processes like coagulation, cell adhesion, wound healing etc.
Heparin

Heparin is a highly sulfated form of heparan sulfate, whereas HS may have an average molecular weight of 50,000 – 100,000, heparin displays an average molecular weight of 15000. High levels of sulfate groups in heparin allow for electrostatic interactions with numerous proteins, thus accounting for its diverse roles in the body, ranging from inhibition of haemostasis to angiogenesis. In fact, an unfractionated mixture of heparin, called unfractionated heparin (UFH), is clinically used as an anticoagulant drug.

![Representative structure of unfractionated heparin (UFH)](image)

A specific five residue sequence, called heparin pentasaccharide, was identified as the functional unit that recognized coagulation enzyme inhibitor, antithrombin (AT). Due to its effective activation of AT and subsequent indirect inhibition of thrombin, heparin has been popularly used as an anticoagulant since the 1920s. It is being gradually been replaced by other anticoagulants such as low molecular weight heparins (enoxaparin, tinzaparin), and pentasaccharide (fondaparinux, etc), due to fatal side effects associated with its administration. The drawbacks of heparin are (1) it can only be administered parenterally (2) binding to plasma, endothelium, platelets, macrophages etc, resulting in unpredictable clearance and a narrow therapeutic window. This causes side effects such as bleeding, thrombocytopenia etc. Such potentially fatal side effects
associated with traditional anticoagulation therapy have led to an unprecedented demand for an “ideal anticoagulant”, especially since deep vein thrombosis, a coagulation disorder, is one of the leading causes of deaths in the world.\textsuperscript{13-18}

The presence of sulfate groups can be seen as a characteristic feature in most of the GAGs discussed above. These sulfate groups, as providers of a highly negatively charged surface have been implicated in GAG recognition and binding to diverse ligands in the body.\textsuperscript{2,8,19} If present in excess, as in case of over-sulfated chondroitin sulfate (OSCS), a heparin contaminant, can cause severe adverse effects.\textsuperscript{20} In 2008, 81 people died to excessive bleeding and several were left in critical state after administration of certain batches of heparin from China which were later found to be contaminated with OSCS.\textsuperscript{21} Hence, characterization of GAGs and determination of degree of sulfation and sulfation pattern is crucial to not only understand the complex interactions of GAGs in the body and optimize them to give least side-effects but also identify contaminants, helping prevent further fatalities.

**Characterization of GAGs**

In a 2011 review on heparin significance and characterization, Jones et al. described heparin structure analysis as composing of roughly three steps.\textsuperscript{22} The first step is depolymerization of unfractionated heparin into small units such as disaccharides and oligosaccharides via enzymatic or chemical cleavage, second, separation of oligosaccharide chains by means of techniques like high pressure liquid chromatography (HPLC) and capillary electrophoresis (CE) and lastly, identification of each separated component by mass spectrometry (MS), tandem mass spectrometry (MS/MS), or nuclear
magnetic resonance (NMR). Hyphenated methods such as HPLC-MS, UPLC-MS, HPLC-NMR, and CE-NMR can be used to perform the last two steps together in a single run and several studies implementing hyphenated techniques in characterization of heparin oligosaccharides (OGs) and low molecular weight heparins (LMWHs) - have been extensively reported in literature. 2-4,22

**Step 1: Depolymerization**

In the first step, GAGs are freed from their PGs by hydrazinolysis, followed by N-acetylation of amine-containing groups. The resultant free GAGs are structurally complex chains that need to be broken down or depolymerized to make analysis less complicated.23 Greater the extent of GAG depolymerization, shorter is the product chain length i.e. complete depolymerization of heparin gives disaccharide units as the final product. These disaccharides could be of eight different types 24 (Figure 1.3) and their compositional analyses have been reported using MS and MS/MS techniques. On the other hand, partial depolymerization results in formation of oligosaccharides, the structure analyses of which are more complex and have been reported in literature using the above mentioned techniques, with varied degrees of success.22,25,26
Enzymatic depolymerization of heparin GAGs involves β (1→4) bond cleavage by bacterial heparinases (or heparin lyases) I, II and III, which differ from one another in terms of cleavage sites and site-specificity (see Figure 1.4). Heparinase III is specific for GlcNx (1→4) y, where x = S or Ac and y = IdoA or glucuronic acid, and it is the most commonly used enzyme for the depolymerization of heparan sulfate. Chondroitin lyases are used in case of depolymerization of CS and hydrolases such as keratanase are used to cleave keratan sulfate. Structure alteration is observed by the introduction of double bond in the hexuronic acid at the non-reducing end.
Chemical depolymerization involves more than one mechanism, namely, oxidation, reductive deamination and β-elimination (see Figure 1.5). Cleavage between C-2 and C-3 of IdoA or GlcA residues containing vicinal –OH groups is achieved by the addition of oxidizing agents like hydrogen peroxide and Cu(II) acetate/Fe(II) sulfate.\textsuperscript{28} Reductive deamination by nitrous acid is pH dependent and results in cleavage of either N-unsubstituted glucosamine (at pH= 1.5) or N-sulfated glucosamine (at pH= 4.0) to anhydromannose in the reducing terminal.\textsuperscript{29} In the presence of benzyl halide, C-5 carboxylic acid of hexuronic acid residue is esterified and introduction of strong base ensures abstraction of proton from C-5 carbon, leading to ester group leaving and formation of double bond between C-4 and C-5 carbons. The difference between enzymatically-derived oligosaccharides (except GAG hydrolase-derived products) and chemically-derived OGs (except β-elimination products) is that in case of the former, UV absorbance is seen at 232nm (4,5-unsaturation in hexuronic acid residue at NRE) whereas in the latter, UV-active/fluorescent/radioactive labels are attached at the reducing ends of the UV-inactive oligosaccharides (OG), for example, para-nitrophenyl hydrazine, biotinylated dianaminopyridine, boron dipyrromethene (BODIPY-FL) hydrazide etc.\textsuperscript{24, 30-33}
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Step 2: Separation

UFH, LWMHs and heparin oligosaccharides are essentially mixtures of GAG chains diverse in chain lengths, degree of sulfation and sulfation pattern. ²² In order to characterize these molecules, one must resolve the mixture into its components and then
proceed to identifying each component using MS, NMR, MS/MS etc. Direct MS infusion of heparin without separation would result in a mass spectrum displaying high noise levels, limiting the extent of information that can be gathered. Therefore, bulky GAGs like heparin and LMWHs (average molecular weights ranging from 5000 to 15000), are first depolymerized into oligosaccharides or disaccharides which then are further separated or resolved into components based on size, charge, polarity etc before heading to structure analysis of each component using techniques like NMR, MS etc.

Traditionally employed approaches for separation of heparin OGs are capillary electrophoresis (CE), Poly acrylamide gel electrophoresis (PAGE) and high pressure liquid chromatography (HPLC). Size-exclusion chromatography (SEC) may be performed to separate depolymerized oligosaccharide mixture into batches based on size range and each of these batches or those of interest may be subjected to HPLC or CE analysis.

**Capillary electrophoresis**

Capillary electrophoresis (CE) is a highly sensitive separation method that takes account of electrophoretic mobilities of analyte components based on the net charge/mass ratio. Since GAGs are highly negatively charged, CE is widely used in their analysis. It also serves as a useful tool in identifying the presence of contaminants such as OSCS in heparin. The major advantages of using CE are (1) its high resolving power, (2) sensitivity, (3) requirement of small amounts of analyte, (4) ease of switching between normal and reverse-polarity conditions (i.e. changing the direction of migration), (5) simple UV detection, and (6) capability of being conjugated with other structure
determination techniques, e.g., with MS and NMR. Exploiting these advantages of CE, a number of studies on analysis of heparin, HS and heparin disaccharides and their compositional analyses have been performed. GAGs can migrate to the anode in the absence of EOF in acidic or low pH conditions; hence reverse-polarity mode is much favored. Owing to its microheterogeneous nature, heparin does not separate well in CE and a broad peak is obtained. The addition of buffers at high concentrations as background electrolytes, for example, 0.6 M lithium phosphate and 0.85 M Tris phosphate at low pH values 2.8 and 3, respectively, showed improvement of CE profile of heparin. In reverse polarity mode, a broad peak of heparin was resolved so as to separate OSCS contaminants from heparin with limits of detection as low as 0.5 to 0.1%. Karamanos et al. resolved hyaluronan and CS disaccharides into their differentially sulfated (0-3) components in 14 minutes. The analyte was passed through an uncoated fused silica capillary with 15mM sodium dihydrogen orthophosphate buffer at pH 3.0 and -20 kV and components could be detected at attomole levels. In addition to voltage gradient, introducing a pressure gradient between the two electrodes has also aided in heparin oligosaccharide analysis. Ruiz-Calero et al. performed compositional analysis of 8 heparin disaccharides using 60mM formic acid buffer at pH 3.4 at -15 kV and a pressure gradient of 3.45x10^{-3} MPa.

Using normal polarity, Desai et al. successfully separated heparin OGs (di- to hexasaccharides) using 10mM sodium borate buffer + 50 mM sodium decyl sulfate (SDS) at a pH of 8.8 and 20 kV. Pervin et al. separated heparin, HS and CS disaccharides by performing CE with 20 mM phosphate buffer (pH 3.48) without significant peak tailing or the use of multiple buffers, at reverse polarity mode. However,
they also observed that normal polarity mode (using conditions established by Desai et al.) was better than reverse polarity CE of heparin OGs of long chain lengths such as tetradecasaccharides.\(^{42}\) Also using similar normal polarity conditions, Scapol et al. additionally used 50-100 mM triethylamine (TEA) to ion-pair with DS and HS disaccharides and the components in the form of molecule-ion pair adducts were detected at 214 nm.\(^{48}\) Detection methods other than UV have been simultaneously explored to obtain better sensitivity, for example, Chang et al. used laser-induced fluorescence with CE to separate and identify seventeen 2-aminoacridone (AMAC)-labeled GAG disaccharides at attomole level, achieving 100 times more sensitivity than CE-UV.\(^{49}\)

As mentioned earlier, CE can be coupled with MS to obtain resolution of heparin GAGs and structural analysis of each component in a single run.\(^{4}\) Although CE-MS seems like an efficient analytical tool for characterization of heparin GAGs, there are many hurdles to this procedure. The buffer salts optimal for CE resolution are usually non-volatile and highly concentrated, hence are MS-incompatible due to inability to vaporize and form ions in an electrospray MS ionization source. To overcome this issue, sheath liquid or a sheath gas needs to be introduced and several parameters such as temperature, capillary position and voltage, cone voltage, sheath gas flow etc need to be modified in a way such that neither ionization nor analyte structural integrity is compromised.\(^{4}\) Duteil et al. were the first to successfully characterize eight heparin disaccharides using CE-MS. Volatile ion-pairing ammonium acetate buffer was used for CE resolution at normal and reverse polarity modes and MS at positive (50 mM ammonium acetate buffer at pH 9.2, sheath liquid composed of 1:1 water/acetonitrile + 3 mM ammonium formate + 2 mM TEA) and negative mode (530 mM ammonium acetate...
Recent applications of CE-MS include frontal analysis CE (FACE) coupled with MS to study heparin pentasaccharide interaction with antithrombin II. However, due to the above mentioned drawbacks and others such as the requirement of skilled personnel to assemble as well as efficiently operate CE-MS and poor reproducibility of CE itself, the use of this method has not yet achieved mainstream popularity.

CE-NMR is a modern analytical tool that has been successfully used in characterization of dansylated amino acids on a nanolitre scale and serum bilirubins but not much has been done in the structural analysis of GAGs, as yet.

**High-performance liquid chromatography (HPLC)**

HPLC involves the use of high pressures (upto 15000 psi) to drive analyte components along with a mobile phase through a column based on their polarities. In normal-phase chromatography, the column is made of hydrophilic/polar matrix e.g. silica, silica bonded with cyano groups, amino groups etc. and the mobile phase is non-polar, for example, \( n \)-hexane. Reversed-phase columns are packed with non-polar stationary phase while the mobile phase passed through the column is generally polar, wherein polar analytes elute earliest. Since heparin GAGs are inherently polar due to the presence of acidic sulfate groups, reversed-phase chromatography, in the presence of alkyl labels/tags, is more favored than normal-phase chromatography. Besides, other advantages of RPC are better resolution, predictable correlation between molecule shape and retention factor etc. HPLC can also be performed using ion-exchange chromatography wherein molecules are resolved based on charge e.g. strong anion.
exchange chromatography-HPLC (SAX-HPLC). Several GAG characterization studies have been reported so far, using different types of HPLC.

**Ion-exchange chromatography**

This chromatography technique is based on charges carried by the analyte, as a result of which, the analyte would form ionic interactions with oppositely charged functional groups covalently bonded to the stationary phase. Based on the charge type of functional groups in the stationary phase, columns could be anion- or cation-exchangers. In case of GAGs, anion-exchange chromatography is used, where the sulfate groups of the GAGs form ionic interactions with positively-charged groups such as amines bonded to the stationary phase. Anion-exchange could be weak or strong depending on the number of anionic groups bonded onto the column matrix. In one of the earliest reports using anion exchange chromatography, Rice et al. separated di- to deca-saccharides of heparin (obtained via heparinase depolymerization) based on their degree of sulfation (charge-based separation), using SAX-HPLC, after having separated them on basis of size by gel permeation chromatography (GPC). Much later, Bultel et al. separated and identified the degree of sulfation and positions of most sulfates present on six heparin hexasaccharides (obtained via nitrous acid depolymerization) using high-performance anion exchange chromatography coupled with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Structural information was obtained through MS fragmentation (inter- and intra- ring cleavages) of hexasaccharides. Trehy et al. implemented SAX-HPLC and were successful in detecting upto 0.03% OSCS and separating impurities like DS from heparin within 25 mins.
late, weak-anion exchange chromatography (WAX-HPLC) has been used to separate and identify heparin impurities in a relatively time-efficient manner. Limtiaco et al. observed that although ion-pairing reversed-phase liquid chromatography with tributylamine had been in use to separate heparin OGs, it did not help retain heparin, let alone separate impurities. Following this, they resorted to weak anion-exchange chromatography and soon realized that heparin and OSCS were strongly bound to the column and did not elute, hence increased the pH so that less, but still sufficient anions remained protonated to interact with heparin. On-line $^1$H-NMR was used to detect peaks for DS, heparin and OSCS eluting one after another. In a collaborative effort involving seven labs, Hashii et al. were able to separate heparin calcium and heparin sodium from OSCS in less than 20 minutes using WAX-HPLC, at detection limits as low as 0.025% OSCS.

Reversed-phase chromatography (RPC)

Unlike SAX-HPLC, where methods developed for any type of analyte need to be implemented on a single type of column, RPC is a polarity-based method that can be carried out using different types of columns e.g. silica-C1, -C6, -C8, -C10, -C18, ethylene bridged hybrid particle (BEH)-C18 (see Figure 1.6), BEH-amide, BEH-phenyl, high strength silica (HSS)-C18, HSS-phenyl-hexyl, HSS-cyanopropyl, charged surface hybrid (CSH)-phenyl hexyl, CSH-fluoro phenyl etc. This makes RPC more versatile and preferred over other types of chromatographic methods. These columns not only differ in chemistries but also properties like particle shape and size, pore size, pH tolerance etc. rendering analyte specificity to each column type. Unlike normal phase liquid
chromatography, RPC employs a hydrophobic stationary phase and a hydrophilic mobile phase and was introduced to separate hydrophobic analytes, although, it is now the most widely used chromatographic method, for all kinds of analytes.

**Figure 1.6.** Illustration of BEH C-18 column chemistry [waters.com]

RPC of GAGs is faced with issues such as too early elution and poor separation due to their highly polar nature. Pre-column derivatization or tagging of GAGs with a suitable hydrophobic UV-active/fluorescent entity can result in better RPC separation. As mentioned earlier, UV-inactive heparin oligosaccharides obtained via chemical
depolymerization are usually tagged with chromophore or fluorophore labels to aid detection. Some of these tags also help in chromatographic separation owing to their hydrophobicity. Derivatization with 2-aminoacridone (AMAC), pyridyl amine/2-amino pyridine (PA), BODIPY, etc. has been popularly employed in RP-HPLC separation of GAGs.²

Deakin et al. observed that labelling standard HS disaccharide mixture with AMAC resulted in efficient picomolar sensitive RP-HPLC separation on a C18 column and fluorescence detection of eight disaccharides.⁵⁵ Performing compositional analysis using two techniques i.e. AMAC-derivatized RP-HPLC and SAX-HPLC of HS GAGs (from enzyme digested rat liver PGs) showed better resolution and sharper peaks in case of RP-HPLC of AMAC-derivatized GAG disaccharides. RP-HPLC and ESI-TOF MS analysis of AMAC-labelled H/HS GAGs has also been useful in studying depolymerization mechanism of heparitinases such as heparitinase I.⁶² Reductive amination using fluorescent arylamine tags such as PA has been reported in several RP-HPLC studies on glycans.²,⁶³,⁶⁴
Figure 1.7. 2-aminopyridine coupling mechanism proposed by Hase et al.65

Noting that reductive amination is a tedious process involving harsh conditions such as high temperature, low pH etc., which could adversely affect GAG structure in the process, Ramsay et al. synthesized \( O \)-hydroxylamine and \( N \)-hydroxylamine fluorescent tags that utilized mild reaction conditions (room temperature) and solvents (water) to react with oligosaccharides via controlled reductive hydroxyl-amination and form oximes and amines of the saccharides, respectively.66 \( O \)-hydroxylamine were found to be suitable tags for PAGE chromatography since multiple, readily-interconvertible, isomeric (syn-, anti-) oximes were generated, complicating HPLC analysis of heparin monosaccharide, but at the same time, could withstand basic conditions and give efficient separation using PAGE. On the other hand, \( N \)-hydroxylamines were concluded suitable fluorescent tags for RP-HPLC separation and ESI-MS detection of enzymatically-derived GAG heparin and HS OGs (except in case of \( N \)-sulfated GAGs where multiple amination products were obtained).
Other UV-active tags such as 1-phenyl-3-methyl pyrazolone (PMP) (strong UV absorbance at 245 nm)\(^67\) and fluorescent tags; 2-aminobenzoic acid/anthranilic acid (AA)\(^68\) and BODIPY\(^24\) have also been successfully used in RP-HPLC-MS based separation and structural analysis of GAG di- and oligosaccharides.

*Hydrophilic-interaction liquid chromatography (HILIC)*

HILIC is a liquid-liquid chromatographic method that separates analytes on basis of polarity. However, in contrast to RPC, the HILIC stationary phase is usually polar and the mobile phase is made of organic or aprotic solvents like acetonitrile, tetrhydroformamide etc containing a small amount of water. This water is believed to form an aqueous environment on the polar stationary phase, resulting in the partitioning of analyte between the stationary phase water layer and the organic mobile phase based on its charge and resulting solubility i.e. greater the polarity, higher is the retention.\(^69\) Using this method, GAGs of different charges, i.e. neutral and acidic, can be separated and analyzed. Derivatization methods such as reductive amination are employed prior to HILIC analysis that uses an amine-bonded stationary phase since the amines (of column) could react with aldehyde groups of sugars (at RE) and form Schiff base.\(^5\) Commonly used columns in the HILIC-based separation of \(\Delta\)-unsaturated GAG di- and oligosaccharides are the amine-bonded and amide-bonded silica columns. As early as 1979, Lee et al. were able to separate and identify three chondroitinase-digested CS disaccharides i.e. \(\Delta\text{Di}-0\text{S}, \Delta\text{Di}-4\text{S}, \Delta\text{Di}-6\text{S}\) in 10 minutes at 100 ng LOD, using a LiChrosorb NH\(_2\) column.\(^70\) Oguma et al. subjected HS (from mouse liver, brain and tumor tissues) to lyase digestion to get \(\Delta\)-unsaturated disaccharides and used amino-
bonded HPLC column to successfully analyze differential compositions of these HS-derived saccharides. They used the same amine-bonded column to perform HILIC-MS/MS compositional analyses of Δ-unsaturated disaccharides of KS found in different bovine parts. i.e. nasal cartilage, brain, cornea and found differences in C6 galactosamine degree of sulfation (nasal cartilage> cornea> brain). When HILIC is combined with on-line UV/MS identification techniques, salts such as ammonium formate, sodium dihydrogen phosphate etc. are often added as solvent modifiers to increase sensitivity and compatibility. Alternatively, amide columns have also been used, as they are less basic and do not form Schiff bases with analyte GAGs. Amide column HILIC combined with negative mode MS was used as an assay to confirm sulfation pattern specificity in AT III binding with HS. HS hexasaccharides of known sulfation patterns were analyzed using HILIC-MS and two of the saccharides were found to bind to AT III, confirming the use of this method as a useful assay to study protein-GAG binding and specificity.

**Step 3: Detection**

**Nuclear magnetic resonance (NMR)**

NMR is a characterization tool that takes into account the spin frequencies of protons or carbon atoms of the analyte to give structural and conformational data. Analyte proton (\(^1\)H) and carbon atom (\(^{13}\)C) precessional frequencies in the presence of a magnetic field (chemical shifts) are a property of their position relative to other atoms in an analyte, an important property exploited in NMR. In case of characterization of heparin GAGs, the use of NMR is faced with a number of issues such as: requirement of
(1) relatively large amounts of sample, (2) high purity of sample, (3) absence of buffer salts or ion-pairing agents etc.\textsuperscript{4,22}

Single dimension (1-D NMR) is useful in limited compositional analysis of heparin GAGs. N-acetylation of glucosamine residue (GlcNAc) in a GAG hexasaccharide was first reported by Pervin et al, using 1-D $^1$H-NMR data.\textsuperscript{34} Following this, they went on to successfully analyze bond connectivities in heparin-derived saccharide by performing two-dimensional NMR (2-D $^1$H-$^{13}$C NMR), obtaining a more thorough picture of GAG structure.\textsuperscript{74}

Uronic acids in GAGs, especially iduronic acids, cannot be reliably quantified using traditional methods like HPLC due to their decomposition into iduronolactones via chemical depolymerization. IdoA and GlcA compositions of HS, DS and heparin from mammalian and porcine sources were analyzed and reported in 2001 by Sudo et al, using 3-step solvolysis (N-desulfation, N-reacetylation, followed by O-desulfation of galactosamine residues) and 2-D NMR.\textsuperscript{75} The solvolysis step eliminated unnecessary signals arising from sulfate groups of GalpNAc residues, thereby helped clearly identify differential 2-D NMR signals from IdoA-GalpNAc bonds and GluA-GalpNAc bonds.

Deakin et al. reported the nature of HS and DS interactions with hepatocyte growth factor/scatter factor (HGF/SF) using NMR.\textsuperscript{76} Using gel mobility assay, they had previously established the requirement of a disulfated IdoA-containing tri/tetra saccharide unit for HS or DS binding interaction with HGF. Performing NMR experiments on $^{15}$N-labeled HGF variant, NK1, titrated with HS and DS oligosaccharides, they observed that
both HS and DS bound HGF at the same binding site, which proved to be fairly flexible and independent of sulfation pattern on either GAGs.

Today, 1-D and 2-D $^1$H-$^{13}$C NMR are analytical tools widely used by pharmaceutical industries manufacturing heparin and LMWHs, to analyze batch purity and identify contamination, if any.$^{77,78}$ This application of NMR gained prominence after its successful use in detecting oversulfated CS (OSCS) in adulterated batches of raw heparin imported from China, which resulted in more than 80 deaths in 2008.$^{79}$

More recently, heteronuclear single quantum coherence spectroscopy (HSQC) $^1$H-$^{15}$N NMR is being investigated as a tool to characterize heparin OGs, based on their N-sulfo-glucosamine (GlcNS) sulfamate residue content and relative position. In 2013, Langeslay et al. reported pH and temperature-controlled $^1$H and $^{15}$N chemical shifts in heparins from various sources and LMWHs such as enoxaparin. Similarly, chemical shifts in various depolymerized heparin OG standards (differing in GlcNS sulfamate position relative to the NRE as well as its degree of sulfation) were obtained and both these data were compared to successfully correlate GlcNS microenvironments with $^1$H-$^{15}$N HSQC peaks originating from different heparins and their oligosaccharides.$^{80}$

Although NMR is an efficient tool for characterization and can be coupled with various separation techniques such as CE, SAX, HPLC etc, the above mentioned disadvantages as well as need for skilled workers to perform experiments and interpret data pose major hurdles in its day to day applicability.$^{4,22}$

**Mass spectrometry**
As the name suggests, “mass spectrometry” is a technique that reveals the identity of an unknown compound by giving its accurate mass information. In case of a mixture of unknown analytes, their signal intensity on a mass spectrum reflects their quantities relative to the greatest ionizable component, usually the solvent. This method of detection is applied in diverse areas, be it in the analysis of progress of a chemical/enzymatic reaction,\textsuperscript{81,82} or to identify drugs of abuse from bodily fluids in picomolar amounts.\textsuperscript{83} In contrast to NMR, MS does not require large amounts of sample nor does it require highly skilled labor to set up or operate.\textsuperscript{2} It is a fairly flexible technique that has been successfully coupled with majority of separation techniques such as CE, HPLC, SPR, GC etc. and can be set up to run more than 1000 samples per day, generating desired results in a rapid and convenient manner.\textsuperscript{84} Therefore, MS finds its utility in qualitative and quantitative analysis of simple molecules as well as complex mixtures like heparin GAGs. MS involving multiple transitions of parent ions into daughter ions and further on, in other words, tandem MS, is an important tool in GAG composition profiling and holds great promise in GAG sequencing.\textsuperscript{2}

In the field of GAG characterization, a lot of MS work has been reported over the last 2 decades with a handful of scientific review articles that discuss significant developments in this field of research.

General obstacles faced in MS of GAGs are degradation of analyte in the source and subsequent loss in intensity of molecular ion, for example, in hard ionization MS techniques such as electron ionization (EI) and fast atom bombardment (FAB).\textsuperscript{2} Also, in soft ionization MS techniques such as matrix-assisted laser desorption ionization
(MALDI), in addition to fragmentation, GAGs have been observed to interact with the ions in matrices due to their highly polar nature, leading to noisy cationic peaks. Several studies have reported breakage of glycosidic linkages between GAG saccharides and more often, loss of acidic sulfate groups during ionization, formation of cationic (K\(^+\), Na\(^+\)) complexes, resulting in a molecular ion of altered identity. Attempts at structure elucidation or sequencing by tandem MS of such a parent ion cannot be fruitful. Hence, much research has been directed towards overcome this problem, resulting in techniques such as ion-pairing, derivatization, peptide-linkage of GAGs etc.

In 1994, Juhas and Beimann reported the use of peptides to characterize complex molecules such as heparin GAGs. By using basic synthetic peptide SP 3 (IRRERNKMAAAK-SRNRRRELTDTL; molecular weight – 2942.41), and mixing it with octasulfated heparin hexsaccharide in 1:1 ratio in a sinapinic acid matrix, they were able to successfully eliminate metal counterion signals and obtain molecular ion peaks. The peaks showed loss of 1 to 3 sulfate groups, but resultant data was at least indicative of the saccharide content and minimum number of sulfate groups. Further developments in characterization studies of GAGs using MALDI led to changes in the matrix, such as use of ionic liquid matrices, introduction of co-matrices such as quaternary ammonium salts (Ueki et al) etc. These developments helped eliminate unwanted metal K\(^+\), Na\(^+\) adduct ion signals and reduce sulfate losses to a greater extent.

In addition to MALDI, several reports indicate the use of electrospray ionization (ESI) MS for GAG structure determination, sequencing, composition determination etc. In ESI, small amounts of sample are mixed with a volatile solvent and
introduced into the source in the form of an aerosol spray. High voltage and temperature conditions transform the sample into gaseous singly-charged or multi-charged ions, which appear on the spectrum as mass/charge peaks. The principle of ESI will be discussed in the next section (UPLC-MS).

ESI-MS-MS involves shooting the parent ion with high energy collision gas, causing it to fragment into distinct daughter ions, in a reproducible manner (see Figure 1.8 for ESI-MS-MS instrumentation).

**Figure 1.8.** Schematic of an ESI-MS-MS triple quadrupole spectrometer. (Snapshot taken from MassLynx 4.1, Acquity TQD mass spectrometer console)

In case of GAGs, fragmentation can result out of glycosidic bond cleavage, cross-ring cleavages and sulfonate cleavage. Sulfate losses tend to occur more abundantly than
other cleavages, resulting in loss of structural information. Ion-pairing these sulfate groups with calcium ion has been reported to make GAG sulfonate groups less prone to fragmentation than as a sodium or potassium salt. \(^{87}\) ESI-MS, in general, and even MALDI, always result in generation of even electron species. A novel route to ensure more cross-ring cleavages than sulfate loss, upon MS-MS, is electron detachment dissociation (EDD) fragmentation, wherein shooting the parent ion with a beam of electrons, results in the generation of an odd-electron species (by the loss of a single electron). \(^{88-90}\) Heparin oligosaccharide sequencing tool (HOST), developed by Saad and Leary, is capable of automatically generating sequence information from MS-MS data, but fails to differentiate between uronic acid epimers. \(^{91}\)

Overcoming this drawback, Wolff et al., in 2007, reported the application of EDD in Fourier transform ion cyclotron resonance (FTICR) MS. This method resulted in generation of cross-ring and glycosidic bond cleavages in odd electron parent species, making it possible to identify sulfation sites as well as differentiate between iduronic and glucuronic acid residues in heparin tetrasaccharides. \(^{92}\)

Composition analysis of heparin disaccharide mixture using CID MS\(^n\), has resulted in identification and quantification of all of the twelve isomers that are generated from heparin-lyase digestion (see Figure 1.9 ). \(^{93}\) In order to prevent metal adduct ion peaks, ammonium hydroxide was introduced into the 1:1 water: methanol solution containing heparinase-digested mixture. MS\(^n\) of this mixture generated diagnostic daughter ions from each disaccharide in reproducible intensities. Such fingerprints are
projected to be instrumental in compositional profiling of longer chain heparin oligosaccharides.

Figure 1.9. Chemical structures of the 12 disaccharides formed by heparinase I, II, and III digestion of heparin and HS are described by the pairwise combination of a roman numeral and a non-italicized letter from the table (e.g. IS ≡ ΔUA2S-GlcNS6S). Propionyl groups designated by the italicized ‘P’ can be added chemically to generate synthetic standards. (Image adapted from Behr et al. 2005).93

<table>
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<th>Number</th>
<th>(R_1)</th>
<th>(R_2)</th>
<th>Series</th>
<th>(R_3)</th>
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<tbody>
<tr>
<td>I</td>
<td>SO(_3)H</td>
<td>SO(_3)H</td>
<td>A</td>
<td>Ac</td>
</tr>
<tr>
<td>II</td>
<td>H</td>
<td>SO(_3)H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>III</td>
<td>SO(_3)H</td>
<td>H</td>
<td>S</td>
<td>SO(_3)H</td>
</tr>
<tr>
<td>IV</td>
<td>H</td>
<td>H</td>
<td>P</td>
<td>COEt</td>
</tr>
</tbody>
</table>

Owing to its versatility, MS is widely used, not just alone, but also in combination with various chromatographic methods (reversed-phase LC, ion-pairing reversed-phase LC, SEC etc) in the structure elucidation of molecules as complex and microheterogeneous as heparin GAGs.

**LC-MS of heparin GAGs**
Both liquid chromatography and mass spectrometry of GAGs have already been discussed, in detail, separately, in the previous sections. There are several reports in literature that utilize LC-MS for characterization of GAGs.\textsuperscript{2,4,22} This is mainly because both these techniques can be easily hyphenated, relatively easy to operate, highly sensitive (upto picomolar sensitivities have been reported) and together, they can be used to generate large amounts of data in a single day. For applications, refer back to high performance liquid chromatography (HPLC).

Since in this project, reversed-phase ion-pairing LC-MS was used as our characterization tool of choice, the principle, working and applications of this method will be discussed in detail, from here on.

**Reversed phase ion-pairing liquid chromatography (RPIP)**

RPIP is a method of RPC where hydrophobic ‘hetaerons’ or ion-pairing agents, oppositely charged to the analyte molecule of interest, are introduced into the mobile phase in order to form ion-pairs with the target analyte. Formation of these ion-pairs renders the analyte hydrophobic enough to retain on the column for longer times (retention factor between 5 and 20).\textsuperscript{94}

**Retention factor ($k'$) :** This is a simpler, conventional way of representing the retention of an analyte in a column than retention time alone. Retention factor takes into account the time it takes for the column to flush out the solvent and deducts it from the time it takes for the analyte to elute out of the column (see Figure 1.10). The efficiency of an LC method/system can be measured by $k'$ of the analyte in given column environment.
Ideally, $k'$ value should be greater than 5 and less than 20. Any analyte that shows a $k' < 5$ is poorly retained on the column and if its $k' > 20$ then it is retained too strongly in the column.

![Diagrammatic representation of a typical liquid chromatogram](image)

**Figure 1.10.** Diagrammatic representation of a typical liquid chromatogram

Retention factor,

$$
k' = \frac{(t_{\text{analyte}}) - (t_{\text{solvent}})}{(t_{\text{solvent}})}
$$

Where,

$t_{\text{analyte}} = \text{time taken for the analyte peak to appear on the chromatogram}$

$t_{\text{solvent}} = \text{time taken for the solvent peak to appear on the chromatogram}$

Acidic molecules such as heparin GAGs, owing to their hydrophilicity, are poorly retained on an RP column, showing $k'$ values < 2. A popular method used to overcome
this hurdle is RPIP chromatography. Positively charged ion-pairing agents, such as quaternary tetraalkylammonium phosphate has been traditionally used in RPIP LC-MS characterization of heparin GAGs and LMWHs.\textsuperscript{95,96} Unfortunately, with time, quaternary amines have been found to be detrimental to the mass spectrometer and can reduce the signal-to-noise ratios as they are less volatile in nature.\textsuperscript{95,97}

**Ion-pairing**

Two kinetic mechanisms have been proposed as involved in ion-pairing.\textsuperscript{98}

1. Interaction of analyte with ion-pairing agent in the mobile phase and subsequent adsorption of the analyte-ion pairing agent complex onto the stationary phase.

2. Adsorption of the ion-pairing agent onto the stationary phase, owing to hydrophobicity from alkyl chain(s) of the agent. Analyte then binds to this hydrophobic charged stationary phase due to attractive forces between unlike charges, resembling ion-exchange. Only **peak widths** (and not **retention**) are affected by the two mechanisms.\textsuperscript{98}

During ion-pairing, the column is in a state of dynamic equilibrium, governed by several factors.\textsuperscript{97-99} These thermodynamic factors/processes are in turn believed to affect **retention** in RPIP:

1. Net charge on the column stationary phase; this is the amount of ion-pairing agent, P, adsorbed onto the stationary phase. It is directly dependent on the concentration of P in the column and its chain length. Greater the concentration/chain length, more saturated the column is, resulting in greater retention of charged analyte.
2. pH; affects RPC and IPRP differently. In case of RPC, an acidic molecule (eg. RCOOH), at low pH, would be retained greater (unionized form, RCOOH, is hydrophobic and sticks to the stationary phase by means of hydrophobic-hydrophobic attraction) and at high pH, the ionized RCOO⁻ form would elute out with less or no retention. On the other hand, in IPRP, the stationary phase is now a charged dynamic ion-exchanger. At low pH, unionized acidic molecule would elute early/be less retained, whereas at high pH (pH > pKa of acidic molecule), deprotonated acidic analyte ions would be attracted to oppositely charged ion-pairing agent-coated stationary phase and therefore be retained.

3. Amount of analyte:P complex in the mobile phase; dependent on strength of organic mobile phase and concentration flowing through the column. In general a gradient elution with increasing organic phase, increases retention, however, if there is too much organic phase or if it is of higher strength, then the organic mobile phase may actually compete with the hydrophobic stationary phase in their ability to carry analyte:P complex. As a result, retention may actually decrease after a plateau is reached.

4. Other factors such as buffers, temperature of the column; buffers help control the pH and keep the ion-pairing agents as well as the analytes charged. Reproducibility of separations requires constant monitoring and regulation of column temperature.

**Electrospray ionization mass spectrometry (ESI-MS)**
ESI-MS is an MS technique introduced by Dr. John Fenn,\textsuperscript{101,102} for which he was awarded the Nobel Prize for chemistry in 2002. Electrospray is the phenomenon of liquid dispersion as a spray of fine droplets, in other words, an aerosol, under the influence of an electric field. As a concept, electrospray had been explored more than a century ago by Lord Rayleigh when he proposed the Rayleigh limit for ionic droplets. However, it wasn’t until Fenn’s invention, that this phenomenon was used in mass spectrometry. Today, a word search on “ESI-MS” gives 10186 hits on PubMed alone, an evidence of the popularity of this revolutionary technology.

While MALDI has no limitation on the mass that can be analyzed, ESI does possess a mass limitation (eg. 2048 Da). However, it still can be used to analyze macromolecules of high molecular weights, as ESI tends to form multiply charged ions. Some of its reported applications in structure analysis of GAGs are listed in the previous section - Mass spectrometry.

**Electrospray ionization – principle**

Although some extent of what happens in electrospray ionization is still a mystery to scientists, it is known as a combination of electrostatic, electrochemical, and thermodynamic processes occurring simultaneously.\textsuperscript{100,102-105}

As the analyte, mixed with volatile solvent, is infused into the MS source through the injection needle, it flows out through a fine capillary maintained at high voltage (about 2 – 4 kV) and atmospheric pressure. Such conditions, along with high temperature (250 – 450 °C) and nitrogen gas flow (at about 650 L/hr), cause the solvent to evaporate
and analyte to be ejected as spray of ionized droplets. In case of positive mode MS, oxidation occurs at the capillary tip, leading to generation of positively charged ions, by the loss of electrons. This dynamic electrochemical process is inverted in case of negative mode MS. The aerosol spray of ionic droplets ends up as fine singly or multiply charged gaseous ions, following any one of the two hypothetical mechanisms explained below.

1. Charge-residue mechanism:

   The droplets each contain several analyte ions. As they evaporate due to high temperature and nitrogen flow, the surface tension of droplets increases, to a point where they can no longer sustain repulsive forces within, and explode into fine droplets. This is called Rayleigh limit. Each of these fine droplets contains one or more analyte ions (charges). Upon further coulombic fission, ultra-fine droplets are formed, remaining solvent evaporates and the analyte ions enter gas phase. This mechanism is said to be followed by analytes of high masses.

2. Ion-evaporation mechanism:

   Ultra fine droplets are formed by coulombic fission, just like in charge-residue mechanism. Contrarily, however, analytes do not undergo repetitive fissions and solvent evaporations to give rise to gaseous ions. In fact, the electric strength of the fine droplets reaches a high due to the decrease in size, making it favorable for the analyte ions to move from droplet surface, into gaseous phase, to be stable. This mechanism is believed to be followed by lower mass analytes.
In a typical ESI-MS ionization chamber, the aerosol spray is 90 degrees to the skimmer cone, allowing for neutral species to go into waste, instead of being pulled into the MS, by means of the cone. The skimmer cone is maintained at voltage that can be manually altered. This voltage difference between the capillary and the cone is another factor that contributes to the electrospray formation. At the same time, this voltage difference as well as that between the skimmer cone and the quadrupole, causes fragmentation of ions and subsequent loss of structural information. This is known as up-front collision-induced dissociation (CID), explained in detail in chapter four.

Figure 1.11. Pictorial representation of Taylor cone formation and 'dragging' of the ions formed through a potential gradient (an electric field) to the counter plate. (Image courtesy of Andreas Dahlin (www.adorgraphics.com))
Ion-pairing reverse-phase LC-MS of GAGs

Ion-pairing reversed-phase LC-MS has been widely reported in literature as a preferred analytical tool for the structure analysis of complex glycan mixtures such as heparin GAGs. From as early as 1980, quaternary amines have been studied as agents that could potentially ion-pair with and separate heparin disaccharides. Several literature reports show the use of tetraalkylammonium and trialkylamine salts such as tetrabutyl ammonium phosphate and tributylamine for the characterization of heparin, LWMHs and heparin oligosaccharides. Quantitation of eight disaccharides in heparan sulfate and heparin was done using IP-RP microflow HPLC-MS and 12 mM tributylamine as the ion-pairing agent. Using radiolabeled glycans as internal standards and tandem MS, the quantities of each heparin/ HS disaccharide were calculated and compared with mixtures of known disaccharide composition, finding no more than a difference of 2 ng between the two. Furthermore, disaccharide quantities and composition in HS obtained from different sources, such as, porcine liver, bovine brain were found, following the same procedure. Thanawiroon et al. used partially (30%) digested bovine lung heparin to obtain oligosaccharides of varied chain lengths. Using 15 mM tributylamine along with 50 mM ammonium acetate buffer, they obtained resolution of up to di- to tetradeca- saccharide length, under negative MS mode, and peaks of up to dp28 length, using positive mode MS.

However, non-volatile ion-pairing agents such as tetraalkylammonium cation, sulfonate and phosphate anion have been observed to cause mass spectrometric pollution, resulting in significant drop in MS sensitivity and reduced signal to noise ratio.
Hence, despite their popularity, the use of these agents had to be cut down and other agents were investigated. Kuberan et al. found that dibutylamine at 5mM, in comparison with other secondary, tertiary and quaternary amines, was optimal for characterizing heparin oligosaccharides.\textsuperscript{111} An ideal ion-pairing agent should not only easily ion-pair with analyte, but also be volatile enough to not block the MS source during spray formation.

In other efforts, quaternary and tertiary amines were continued to be used as ion-pairing agents, but methods to get rid of these counter ions before they reach the MS source, were being pursued. This involved using ion-trap MS, introducing sheath liquids, valve switching, addition of volatile additives etc. Loss of analyte signal, requirement of specialized equipment and skilled personnel etc. are some of the drawbacks of using these methods.\textsuperscript{97} Additives may cause structural and chemical changes in the analyte molecule, in turn affecting column retention as well as MS ionization of analyte.

**Rationale**

In the Desai lab, we aim to discover small molecule mimics of heparin, by synthesizing on a high-throughput scale, as a positive, much-needed step towards safer anti coagulation therapy. Therefore, the characterization of these heparin mimetic molecules is imperative to further our research in coagulation therapy. There are, however, challenges in this direction.

As noted previously in this chapter, heparin oligosaccharides not only retain minimally in reversed-phase columns but also tend to lose structural information in the
MS, by means of sulfonate losses that occur before inter and intra-ring cleavages. Similar behavior is exhibited by our mimetic compounds, since they are also sulfated.

In addition, these sulfated mimetics, owing to their high hydrophilicities, are not retained on reversed-phase HPLC column, similar to heparin GAGs. NMR analysis requires large amount of sample, does not show the presence of sulfates directly, and is not sensitive to small amounts of degradation products in the sample.

Literature reports, in the last few decades, indicate that ion-pairing LC-MS has shown much promise as the analytical tool of choice in heparin GAG characterization. Hence, ion-pairing reversed-phase UPLC-MS was investigated as a method to characterize to our library of heparin mimetics. Linear and tertiary amines were tested as IPAs, against a library of active molecules, belonging to diverse scaffolds. We examined the effect of each IPA on analyte retention, as well as, on its subsequent ionization. These results laid foundations to understanding the mechanism of ion-pairing and the factors influencing it.

Also, unique fingerprints of each compound, generated using MS alone, were useful in differentiating positional isomers. Further studies may, perhaps, help us in obtaining more structural information, such as the relative positions of sulfate groups in an unknown analyte/mixture.

Agents that gave maximum retention and preserved the analyte’s structural integrity with no compromise in MS signal intensity, were chosen for characterization of larger, complex biologically active mixtures synthesized in our lab.
The focus of this work is the characterization of heparin mimetics by achieving optimal retention and MS ionization of intact molecular ions, without ion suppression. At the same time, in order to develop a method that is reliable, sensitive and easy-to-use on a high-throughput scale, we ensured that no pre-column or post-column modifications are required. The RPIP UPLC-MS method aims to be useful in the characterization of larger, highly sulfated mixtures, without the requirement for depolymerization or permethylation of sulfate groups prior to LC-MS analysis.
CHAPTER 2

Development of Reversed-Phase Ion-Pairing Liquid Chromatography-Mass Spectrometric Analysis Of Catechin Sulfate

In 2002, the Desai lab published a study on the design of the first non-saccharide activator of antithrombin (AT), known as epicatechin sulfate (ECS). ECS, a flavanoid with five sulfate groups, was reported to exhibit a strong affinity for AT with $K_d \sim 10.7 \mu M$. In order to understand its binding mode, structurally similar sulfated flavanoids i.e. (+) catechin sulfate (CS) and (±) catechin sulfate (RCS) were studied. Competition assays and molecular docking studies showed that ECS, CS and RCS competed with heparin to bind AT at the extended heparin binding site (EHBS) via ionic interactions.

Figure 2.1. (+)-catechin sulfate (CS); (-) - epicatechin sulfate (ECS)

CS has since been pursued as a molecule of interest for the following reasons; (1) it exhibits heparin mimicking properties; (2) it is non-saccharide based; (3) it has five sulfate groups, imparting charge density similar to heparin pentasaccharide (4) is homogeneous and hence, is expected to exhibit considerable specificity in its interactions, unlike heparin and LMWHs.
Several methods have been employed to characterize CS in the past few years, each with limitations of its own. In a reverse polarity capillary electrophoretic CS analysis by Dantuluri et al., separation of flavanoids (CS and ECS) from flavonoids (quercetin and apigenin sulfate) was achieved; however no mass identification was attempted.¹¹³ This CE method can be used only when the identity and degree of sulfation of sample components are known. Disadvantages such as low reproducibility, mar the use of CE alone as an analytical tool. With reversed phase LC-MS, CS is not retained on either UPLC or the HPLC column due to its high polarity. In addition, MS in-source sulfonate losses (as SO₃⁻- Na⁺ ions) result in mass spectra containing numerous ion signals, making it difficult to deduce any useful information.

Although CS is structurally similar to GAG saccharides in being polysulfated, it shows one major difference; it possesses non-saccharide based, i.e., its sulfates are aromatic (phenolic), and not aliphatic, as in GAGs (see Figure 2.1). Hence, sulfates in CS are relatively more labile due to the tendency of a phenolic sulfate to lose sulfonate and form phenolic-OH by resonance stabilization. This makes the characterization of CS without pre-column derivatization or inclusion of protecting additives, such as sheath gas etc. in the MS, an arduous task.

In contrast to the aforementioned methods, RPIP-UPLC-MS allows for analysis of compounds without the necessity for pre- or post-column modifications. It is a highly-sensitive method requiring very small amounts of sample and has been well reported in the analyses of highly sulfated structurally complex heparin and HS oligosaccharides.
Traditionally used ion-pairing agents are alkyl sulfates (negatively-charged ion-pairing agents) for the retention of positively-charged analytes and tetraalkylammonium phosphates (positively-charged ion-pairing agents), in case of anionic analytes. In this study, we used an array of primary (linear) and tertiary (branched) amines as ion-pairing agents and tested each of them in concentrations ranging 5 to 50 mM to find the optimal agent and optimal concentration for UPLC retention and MS identification of CS.

2.2 Experimental methods

2.2.1. Materials

(+)-catechin sulfate (CS) and (-)-epicatechin sulfate (ECS) (synthesized by the Desai lab and stored in -80°C), ion-pairing agents, i.e., n-butylamine (BTA), n-pentylamine (PTA), n-hexylamine (HXA) of the highest purity were purchased from Fisher Scientific (Fair Lawn, NJ). Tripropylamine (TPA) and tripentylamine (TPentA) of the highest purity, and solvents such as optima LC-MS grade acetonitrile, methanol, were from Sigma (St. Louis, MO). Optima LC-MS grade formic acid was purchased from Fisher Scientific (Fair Lawn, NJ) and Nerl high-purity water from Thermo Fisher Scientific (Middletown, VA).

2.2.2. RP-IP UPLC-MS of Catechin Sulfate

Ultraperformance liquid chromatography (UPLC) was used in conjunction with electrospray ionization-mass spectrometry (ESI-MS) in order to characterize CS. Waters Acquity H-class UPLC system equipped with a triple quadrupole mass spectrometer (TQD-MS) was used to perform all experiments.
A reversed-phase Waters BEH (Ethylene Bridged Hybrid) C18 column of 1.7 µm particle size and 2.1 x 50 mm dimensions was used as the column for all the UPLC experiments. A flow rate of 500 µL/min and column temperature of 40°C was maintained during separation. Solvent A consisted of 5-50 mM ion-pairing agent in water containing 0.1% v/v formic acid (to protonate ion-pairing agent) and solvent B consisted of 5-50 mM ion-pairing agent in acetonitrile–water mixture (3:1 v/v) containing 0.1% (v/v) formic acid.

CS and ECS synthesized in our lab were used (Figures 2.13 and 2.14 are corresponding NMR spectra) and 0.5 mM solutions were made by adding 70 µL of 10 mg/ml of each in 1.5 ml water. 5 µL of this sample was injected onto the column. A 7 minute run was performed at a gradient starting with 20% solvent B, rising linearly to 80% in 3 minutes (20% per min). The flow was then set to isocratic for 2 minutes, followed by a return to initial conditions in the last 2 minutes.

The eluent from the UPLC was directly introduced into the TQD-MS. MS experiments were performed in positive mode as it was found to be better than negative mode in previous studies with heparin oligosaccharides and in our UPLC-MS experiments with CS (Figure 2.12) and other sulfated molecules in general. For MS tuning at positive mode, capillary voltage was set at 4 kV with a desolvation temperature of 350°C and gas flow at 650 L/hr. UPLC-MS method consisted of scans for m/z ranges of 200-2048 with scan time of 0.25 seconds. Selected Ion Recording (SIR) set at a dwell time of 0.025 seconds, was also performed for masses corresponding to singly charged [CS-ion pairing agent] molecular adduct ion; 

\[ m/z = [799.97 - 5 (\text{Na}) + 6 \text{IPA}]^{1+}. \]
2.3 Results and Discussion

2.3.1. RP-IP UPLC-MS Characterization of CS

UPLC-MS of 500 μM solutions of CS and ECS, in the absence of any ion-pairing agents in the mobile phase, resulted in poorly retained single peak eluting soon after solvent peak (0.30 minutes), at 0.33-0.35 minutes on the chromatogram (see Figure 2.4).

Therefore, retention factor (equation explained in chapter 1),

\[ k' = \frac{(0.35 - 0.30)}{(0.30)} \]

\[ k' = 0.16 \]

The peaks in both cases showed a retention factor, \( k' \approx 0.1 \), which is much less than the ideal \( k' \) range of 5-20.

The mass spectra of these CS and ECS peaks did not show the presence of molecular ion, \([M + H]^+; m/z = 800.78\), or sodiated adduct \([M + Na]^+; m/z = 822.76\). Instead, we observed signals that corresponded to \( m/z \) of protonated molecular ion devoid of a single sodium sulfonate group i.e. \([(M – NaSO_3) + H]^+, m/z = 698.3 \) (ppm error ≤ 0.5) at electron count intensities of 195290 (1.9e⁵) and 547273 (5.4e⁵) for ECS and CS respectively. Also found in greater abundance were peaks corresponding to loss of upto
four sulfate groups (see Figure 2.5). These peaks are hypothesized to arise out of structural modifications of analyte molecules in the (a) LC column, (b) MS source.

As the LC run begins and mobile phases flow through the column, 0.5 μM formic acid in the mobile phases forms hydrophobic interactions with the stationary phase via its alkyl carbon. In a fashion similar to ion-exchange chromatography, sodium ions of CS and ECS injected into the column may be dynamically exchanged with the protons of ionized formic acid, modifying the molecule structure (CS or ECS) in the UPLC column (see Figure 2.2).

**Figure 2.2** Dynamic ion exchange in the UPLC column results in at least six inter-convertible forms of CS entering into the MS source.
As six possible forms of CS/ECS enter into the MS source, it is observed that they fragment into smaller molecules due to lability of sulfate groups to high voltage potential and temperature conditions in the source (see Figure 2.3). The presence of metal cations such as sodium seems to do less to protect these groups from cleaving off the molecule scaffold.

Figure 2.3 Three fragment ions (highlighted in yellow) formed in the MS source, from different CS molecules in the column eluate
Mass spectra shows the presence of these three fragment ions as well as many others, representing MS data of poor quality (see Figure 2.5).

In the presence of 5 to 50 mM ion-pairing agents in the mobile phases, UPLC-MS of CS was performed, in both positive and negative MS modes. In both modes and in presence of all the ion-pairing agents used (see Figure 2.6), peak for CS molecular adduct ion, \([(M - 5 \text{ Na} + 5 \text{ IPA}) + 1 \text{ IPA}]^{1+}\), was observed. Retention improved significantly as CS showed \(k'\) values ranging from 0.2 to 11 in the presence of linear and tertiary amines. Figure 2.7 represents a plot of \(k'\) versus concentration of ion pairing agents (mM) where highest retention is observed with tripentylamine and lowest in case of n-butylamine. Optimal retention was observed with linear amines, PTA and HXA as well as tertiary amine, TPA i.e. \(k'\) was observed between 4 and 8. Single ion chromatograms show the presence of well retained CS molecular adduct ion at peak intensities of \(7.86e^6\) and \(2.84e^7\) in presence of hexylamine and pentylamine respectively (see Figures 2.8 and 2.10). Retention times were at 1.53 and 2.04 minutes respectively, resulting in \(k'\) values between 4.1 and 5.8. Corresponding MS spectra show the presence of only the molecular adduct ion peak and no fragment ions, in both cases. Molecular adduct ion peak intensities are high and found to be ranging between \(e^6\) and \(e^7\) (see Figures 2.9 and 2.11).

Figure 2.12 shows the decrease in MS signal intensities of molecular adduct ions, in case of negative mode. Under MS negative mode, in the presence of four ion-pairing agents at 5 to 50 mM concentrations, molecular adduct ion peaks were observed at intensities less than 20 % of those obtained in positive mode MS.

**Linear vs. Branched amines**
Linear amines have been previously reported to give better peak capacities than quaternary and tertiary amines, in the UPLC-MS of heparin oligosaccharides. In case of CS, linear amines showed about ten times higher MS signal intensities than tertiary amines (for singly charged molecular adduct ions); indicating that linear amines due to their single chain structure, have a greater tendency to form adduct ion droplets in the MS source. Tertiary amines, owing to their branched shape, may occupy too much space or may conglomerate together and cause repulsions, preventing the formation of fine droplets in the MS source, during aerosol formation and/or ionization. Also, the use of tertiary amines has showed gradual decrease in overall MS sensitivity, indicating ion suppression associated with their MS application.

On the other hand, tertiary amines gave higher retention factors than linear amines, which can be attributed to the number of alkyl chains i.e. enhanced retention in the column stationary phase (Figures 2.8 and 2.10), as well as increased chances of encountering and ion-pairing with analyte molecules by means of their alkyl chain branches. It could therefore be assumed that greater the number of branches, greater is the retention, but this will soon be proved otherwise in chapter 3, giving us an idea of the complexity of ion-pairing mechanisms that take place in the column.

Positive mode MS of CS in presence of linear ion-pairing agent (IPA) gave molecular adduct ion signal of \( m/z = [(M + 5 \text{ IPA}) + 1 \text{ IPA}]^{1+} \) and branched IPA usually gave molecular adduct ion signal of \( m/z = [(M + 5 \text{ IPA}) + 2 \text{ IPA}]^{2+} \), indicative of the increased propensity of CS to form doubly-charged ions in presence of branched ion-pairing agents. Therefore, we suggest that the type of ions formed could be a function of
physical property such as structure of ion-pairing agent, with linear amines favoring formation of singly-charged ions and branched amines favoring multiply-charged ions.

In addition to positive mode, all the runs were performed in negative mode MS, which resulted in formation of ions of $m/z = [(M + 5 \text{ IPA}) - 1 \text{ IPA}]^{1-}$ and $[(M + 5 \text{ IPA}) - 2 \text{ IPA}]^{2-}$, when ion-paired with linear and branched amines respectively. The MS signal intensities, however, were observed to be reduced, indicating the decrease in ability to form negatively-charged ions.

In order to reach optimal MS conditions, several parameters such as temperature and capillary voltage had to be adjusted; however, the most important was found to be cone voltage. As cone voltage was increased beyond 20 V, a significant number of ion signals cropped up in the MS spectrum, corresponding to in-source fragmentation losses of sulfonates (as sulfonate-ion pairing agent) from the molecular adduct ion. However, these losses were more controlled and the molecular adduct ion was found to be highly stable, relative to when no ion-pairing technique was employed.

At MS cone voltage of 20 V, molecular adduct ion peaks were obtained at highest intensities without any sulfonate losses, applicable across the board for all ion-pairing agents.

Ion-pairing with linear amines helped successfully retain CS on the column and appropriate tuning of MS conditions, specifically cone voltage, resulted in intense molecular adduct ion signals and complete elimination of sulfonate group losses, overcoming both the hurdles using a single technique (Figures 2.9 and 2.11). 25 mM was
found to be the optimal concentration for all ion-pairing agents and HXA at 25 mM was found to be the best agent for UPLC-MS characterization of CS. Positive mode was found to be better ionizing than negative mode in this study.

This method is simple, convenient and eliminates the need for any kind of pre-column derivatization technique like permethylation, or any post-column sheath liquid/gas addition. In comparison to other methods like NMR, this technique requires far less amount of sample (5 μL) and is highly sensitive. Capillary electrophoresis has so far shown limited use as a characterization tool for CS it does not give any identification data despite giving good resolution and pairing CE with MS, as mentioned previously, is a procedure fraught with technical difficulties.

In this work, we used RPIP-UPLC-MS, to successfully characterize CS, without the requirement of any kind of pre-column derivatization or post-column modifications. By utilizing ion-pairing agents, we were able to obtain good retention factors for CS and also observed that these agents imparted stability to sulfate groups of CS molecular adduct ion. MS cone voltage, when set at 20 V, was found to be optimum for less fragmentation and good signal intensity. Fragmentation was observed when the same experiment was conducted using cone voltages above 20 V. At voltages below 20 V, the molecules failed to ionize properly.

After optimizing conditions such as type and concentration of ion-pairing agent, cone voltage etc. we obtained mass/charge information of intact CS, with little or no compromise to its structural integrity. This is the first report on UPLC-MS characterization of pentasulfated catechin sulfate.
Figure 2.4. Chromatograms for \([M - 1 \text{ NaSO}_3 + 1 \text{ H}]^+\) ion of ECS (above) and CS (below), observed \(m/z = 698.3\); Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 27 V.
Figure 2.5. Corresponding spectra for [M – 1 NaSO\(_3\) + 1 H\]^+ ion of ECS (above) and CS (below), observed \(m/z = 698.3\); Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 27 V.
Figure 2.6. Structures of ion-pairing agents i.e. linear and branched amines used in UPLC-MS characterization of CS
Figure 2.7. Plot showing CS retention factor vs. ion-pairing agents at concentrations ranging from 5 to 50 mM
Figure 2.8. Chromatogram for $[M + 1 \text{ HXA}]^+$ ion of CS, observed $m/z = 1298.4$; Solvent A, 25 mM HXA in water + 0.1% v/v formic acid; Solvent B, 25 mM HXA in acetonitrile: water (3:1) + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 2.9. Mass spectrum for [M + 1 HXA]⁺ ion of CS, observed m/z = 1298.4; Solvent A, 25 mM HXA in water + 0.1% v/v formic acid; Solvent B, 25 mM HXA in acetonitrile: water (3:1) + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 2.10. Chromatogram for [M + 1 PTA]⁺ ion of CS, observed m/z = 1213.4; Solvent A, 25 mM PTA in water + 0.1% v/v formic acid; Solvent B, 25 mM PTA in acetonitrile: water (3:1) + 0.1% v/v formic acid; cone voltage, -20 V.
Figure 2.11. Corresponding mass spectrum (below) for [M + 1 PTA]$^+$ ion of CS, observed $m/z$ = 1213.4; Solvent A, 25 mM PTA in water + 0.1% v/v formic acid; Solvent B, 25 mM PTA in acetonitrile: water (3:1) + 0.1% v/v formic acid; cone voltage, - 20 V.
Figure 2.12. Signal intensities of CS molecular adduct ions in the presence of different ion-pairing agent concentrations (5 to 50 mM), at both positive and negative MS modes. Negative mode shows consistently low intense signals (in case of all ion-pairing agents), relative to positive mode MS.
Figure 2.13. $^1$H NMR of CS
Figure 2.14. $^{13}$C NMR of CS
CHAPTER 3

Development of Reversed-Phase Ion-Pairing Liquid Chromatography-Mass Spectrometric Analysis Of Small Molecule Heparin Mimetics of Various Scaffolds

The Desai lab has been investigating and synthesizing sulfated small molecules of different scaffolds such as tetrahydroisoquinolines, isoquinazolinones, benzofurans, flavanoids etc., aiming to discover a suitable scaffold and the optimal number of sulfates required to exhibit heparin mimicking actions. Factor Xa is a serine protease which plays an important role in the coagulation cascade by activating prothrombin to thrombin. Thrombin, thus formed, binds to soluble fibrinogen and activates it into insoluble fibrin. Fibrin, along with Factor XIIIa, is what forms a “clot”. A few of the sulfated tetrahydroisoquinolines generated by our lab have shown potent factor Xa inhibition,\textsuperscript{114,115} while some sulfated quinazolinones have been reported to inhibit angiogenesis.\textsuperscript{116} Most recently, our monosulfated benzofuran dimers were found to be selective allosteric thrombin modulators.\textsuperscript{117,118} These small molecules are of low molecular weight, homogenous and hence, like CS, are predicted to have more specific effects than that of polymeric GAGs, such as heparin and HS.

Since these molecules have demonstrated impressive biological activities, it becomes imperative to develop analytical techniques for use in biological fluids. The ion-pairing UPLC-MS method employed for the characterization of CS was hypothesized to
be useful for these molecules (Figure 3.1) also. Thus, MS conditions were screened to identify optimal method for characterizing a structurally diverse group of GAG mimetics.

Figure 3.1 Structures of sulfated small molecules designed by the Desai lab to mimic heparin; tetrasulfated tetrahydroisoquinolines, 1 and 2; pentasulfate flavanoid, 3; disulfated quinazoline, 4; monosulfated benzofuran dimer, 5.

3.2 Experimental methods

3.2.1. Materials
Molecules 1, 2, 3, 4 and 5 (synthesized by the Desai lab and stored in -80°C), ion-pairing agents i.e. n-butylamine (BTA), n-pentylamine (PTA), n-hexylamine (HXA) of the highest purity were purchased from Fisher Scientific (Fair Lawn, NJ). n-octylamine (OTA), n-nonylamine (NNA), Tripropylamine (TPA) and Tripentylamine (TPentA) of the highest purity and solvents such as optima LC-MS grade acetonitrile, methanol were from Sigma (St. Louis, MO). Optima LC-MS grade formic acid was purchased from Fisher Scientific (Fair Lawn, NJ) and Nerl High-purity water from Thermo Fisher Scientific (Middletown, VA).

3.2.2. RP-IP UPLC-MS equipment and experimental conditions

All RP-IP UPLC-MS conditions used are the same as that mentioned in section 2.2.2., in chapter 2.

3.3 Results and Discussion

3.3.1. RP-IP UPLC-MS analysis of small molecules 1, 2, 3, 4 and 5

In the absence of ion-pairing agent in the system, all compounds, except for benzofuran dimer (molecule 5) were retained on the column for only 0.25 to 0.45 minutes, \( k' < 2 \) (chapter two; Figure 2.4, chapter three; Figures 3.2, 3.4, 3.6, 3.8). In addition, their positive mode mass spectra were found noisy with several ion peaks; indicating extensive fragmentation of molecular ion or \( \text{Na}^+ \) adduct ion (chapter two; Figure 2.5, chapter three; Figures 3.3, 3.5, 3.7, 3.9). As an exception, molecule 5 was retained fairly well, due to hydrophobicity of the scaffold and presence of only one hydrophilic sulfate group (see Figure 3.8).
Molecule 3, catechin sulfate, is discussed in chapter two, where ion-pairing with 25 mM HXA was found to optimal for UPLC column retention and MS ionization of CS molecular adduct ion. Using the same MS conditions and LC gradient, ion-pairing was tested for molecules 1, 2, 4, and 5, each representing small molecule heparin mimetics of a scaffold. In order to study optimal IPA and IPA concentration for each scaffold, RP-IP UPLC-MS experiments were performed using linear amines; BTA, PTA, HXA, OTA, NNA and tertiary amines; TPA and TriPentA at concentrations 5 to 40 mM. All of these ion-pairing agents, at concentrations 5 to 40 mM, gave molecular adduct ion peaks for molecules 1 to 5; for example; using HXA as IPA, Figures 3.10, 3.12, 3.14, 3.16 show the total ion count chromatograms and single ion chromatograms for molecular adduct ions of 1, 2, 4, and 5 in the presence of 25 mM HXA. Figures 3.11, 3.13, 3.15, 3.17 are the corresponding spectra.

Plotting $k'$ vs. concentrations of ion-pairing agents led us to conclude that molecular adduct ion peaks were observed at LC retention times and MS signal intensities dependent on IPA structure and concentrations. To elaborate, Figure 3.18 shows $k'$ values of molecule 1 plotted against increasing concentrations of linear amine ion pairing agents. Retention was observed to increase with increasing order of alkyl chain length of IPA. BTA (alkyl number, n = 4) retains molecule 1 the least, $k' < 1$; whereas NNA (alkyl number, n = 9) retains it the highest, $k' > 14$. Also, within each IPA, retention factors increased as concentrations increased from 5 to 25 mM and proceeded to plateau or decrease from 25 to 40 mM. Similar plots (Figures 3.19 to 3.22) indicate a similar trend of linear amine IPA effect on retention of molecules 2 to 5.
On the other hand, tertiary amines, TPA and TPentA, also gave high $k'$ values between 3 and 11 for molecules 1 to 5. TPentA showed greater retention than TPA for all molecules but at concentrations above 25 mM, was found to be immiscible with the aqueous mobile phase, hence $k'$ values up to only 15 mM TPentA were obtained (Figures 3.23 to 3.27). In case of TPA, plateau was not reached by 25 mM as retention factor values continued to increase at 40 mM, across the board for all molecules.

In order to study the dependence of retention on the degree of sulfation of a molecule and to possibly be able to quantify the number of sulfates of an unknown molecule on basis of such a correlation, we plotted alkyl number/carbon atom number of ion-pairing agents tested versus $k'$ values of molecules 1 to 5 at fixed concentrations; 15 and 25 mM, of ion-pairing agents (Figures 3.28 and 3.29). Slope, $m$, for each molecule was calculated at both concentrations.

For example, at 15 mM IPA, $k'$ of pentasulfated CS (molecule 3) was 0.615 for BTA (n = 4) and 17.1 for NNA (n = 9). Therefore,

$$m = \frac{\Delta y}{\Delta x}$$

$$\Rightarrow m = \frac{17.1 - 0.615}{9 - 4}$$

$$\Rightarrow m = 3.297$$
Similarly, slope values were calculated for molecules 1 to 5 and a correlation was established.

Plotted in Figure 3.28, are the slopes of retention factors \((k')\) of molecules 1-5 at 15 mM ion-pairing agent concentration (carbon atom number 4 to 9). Slope values \((m)\) show direct proportionality with number of sulfate groups on retained molecule. For e.g. molecules 1 and 2 have four sulfate groups each and have similar hydrophobic surface areas and their \(k'\) slope values are found to be 2.88 and 2.91 respectively. Molecule 5, with the lowest number of sulfates \((n= 1)\), shows the smallest slope value of 1.415, whereas CS (molecule 3) containing five sulfates shows the greatest slope value of 3.311. Figure 3.29 provides further evidence to our hypothesis by showing similar results at 25 mM IPA concentration.

Comparison of linear and tertiary amines of the same alkyl number was done by plotting \(k'\) values of analyte against increasing concentrations of ion pairing agents such as NNA and TPA where \(n = 9\). The resultant plot (Figure 3.30) shows higher \(k'\) values in case of linear amine at concentrations 5 mM to 25 mM. At 25 mM, NNA and TPA retained molecule 2 at \(k' \approx 15\) and \(k' \approx 4\) respectively.

**Ion-pairing with linear amines**

All the linear amines of increasing chain lengths; number of carbons/alkyl number = 4 to 9 (BTA, PTA, HXA, OTA, NNA), successfully ion-paired with compounds 1, 2, 4 and 5. Molecular adduct ion was prominently seen in all spectra, with retention factors
increasing with IPA concentration, till 25 mM. Beyond 25 mM, the retention of all compounds remained the same, indicating saturation of column.

BTA showed least retention of all molecules, while NNA showed greatest retention (Figures 3.18 to 3.22), indicating direct dependence of retention factor on alkyl chain length/carbon atom number of ion-pairing agent. Interestingly, the difference between the retaining effect of n and (n + 1) alkyl group containing ion-pairing agents like PTA and HXA was pronounced in compounds with higher number of sulfates. For e.g. at any concentration of ion-pairing agent, it can observed that difference in $k'$ between any two consecutive ion-pairing agents is greatest in pentasulfated compound 3 (Figure 3.20), followed by tetrasulfated 1 and 2, disulfated 4, and lastly compound 5, monosulfated benzofuran showed least difference in retaining effect of ion-pairing agents (Figures 3.18, 3.19, 3.21, 3.22). These results show that ion-pairing is a phenomenon also dependent on analyte charge density. Hence, the selection of an optimum ion-pairing agent is incumbent upon the degree of sulfation of the target analyte. Analytes of lesser charge density would retain optimally with shorter chain length ion-pairing agents.

**Ion-pairing with tertiary amines**

In case of tertiary amines, similar to linear amines, the retention was found to be dependent on charge density of the sulfated molecule under study (Figures 3.23 to 3.27).

Although it was observed that tertiary amines led to higher $k'$ values than linear amines, when linear and tertiary amines of equal carbon atom number were compared, for e.g. TPA and NNA (carbon atom number = 9), it was observed that NNA gave about
four times greater retention (Figure 3.30). This phenomenon indicates that ion-pairing in
the column is strongly dependent on ion-pairing agent properties such as structure,
greater than on the alkyl nature/carbon atom number of ion-pairing agent.

Overall, it can be concluded that the retention of these sulfated small molecules,
in presence of different ion-pairing agents, does not follow any particular pattern based
on analyte properties such as analyte hydrophobicity, number of charges etc. However,
the relative effect of different ion-pairing agents on the molecules seems to be directly
dependent on number of charges carried by the molecule i.e. an analyte with higher
number of sulfates showed greater increases in retention factor, $k'$, with increase in
carbon atom number of ion-pairing agent.

Not surprisingly, plots of retention factors of all compounds in the presence of 15
and 25 mM ion-pairing agent vs. ion-pairing agents in increasing order of carbon atom
number, gave slopes that correlate with degree of sulfation i.e. higher sulfated
compounds such as 1, 2 and 3 gave slope values greater than those of lower sulfated
compounds 4 and 5 (Figures 3.28 and 3.29).

Further studies on a larger library of molecules are predicted to show if slope
values could indeed be directly proportional to degree of sulfation and if so, then the next
step would be to determine if the number of sulfates of the test compound could be
predicted by its retention factor slope values or slope ranges.
In terms of ion-pairing agent properties that affect retention, dependence of $k'$ on structure/shape seemed to be more pronounced than on number of carbon atoms/hydrophobicity.

25 mM HXA was found to give intense distinct molecular adduct ion peaks with no sulfonate losses (Figures 3.11, 3.13, 3.15 and 3.17), at optimum retention factor values for all compounds ($k'$ values between 6 and 12), closely followed by 25 mM PTA (Figures 3.18 to 3.22).
Figure 3.2. Sulfated small molecule 1; full scan (200-1000 amu) (above) and single ion chromatogram (below) for $[\text{M} + 1 \text{ H}]^+$ ion, $m/z = 710.0$; Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1 % v/v formic acid; cone voltage, 20 V.
Figure 3.3. Corresponding mass spectrum of 1 at 0.27 min; \([M + 1 \text{ H}]^+\) molecular ion peak of observed \(m/z = 710.0\); Solvent A, water + 0.1 % v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.4. Sulfated small molecule 2; full scan (200-1000 amu) (above) and single ion chromatogram (below) for \([M + 1 H]^+\) ion of observed \(m/z = 724.0\); Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.5. Corresponding mass spectrum at 0.26 min; [M + 1 H]⁺ molecular ion peak of observed m/z = 724.0 and [M + 1 Na]⁺ molecular ion-sodium adduct peak of observed m/z = 746.0 are highest abundant, followed by sodium sulfate loss peaks (m/z, 600.4; 497.6); Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
**Figure 3.6.** Sulfated small molecule 4; full scan (200 - 1000 amu) (above) and single ion chromatogram (below) for $[\text{M} + 1 \text{ Na}]^{+}$ ion of observed $m/z = 481.7$; Solvent A, water + 0.1 % v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.7. Corresponding mass spectrum at 0.35 min; [M + 1 Na]⁺ molecular ion-sodium adduct peak of observed m/z = 481.7 and [(M – 2 Na + 2 H) + 1 Na]⁺ base peak of observed m/z = 439.9; Solvent A, water + 0.1 % v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.8. Sulfated small molecule 5; full scan (200-1000 amu) (above) and single ion chromatogram (below) for [(M – 1 Na + 1 H) + 1 H]⁺ ion of observed m/z = 580.5; Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.9. Corresponding mass spectrum at 1.5 min; \([\text{M} - 1\ \text{Na} + 1\ \text{H}]^+\) molecular ion peak of observed \(m/z = 580.5\); Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
**Figure 3.10.** Sulfated small molecule 1; full scan (200-1000 amu) (above) and single ion chromatogram (below) for \([\text{M} - 4 \text{Na} + 4 \text{HXA} + 1 \text{HXA}]^+\) ion of observed \(m/z = 1128.07\); Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, 3:1; water: acetonitrile containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.11. Corresponding mass spectrum at 1.88 min; [(M – 4 Na + 4 HXA) + 1 HXA]⁺ molecular adduct ion peak of observed m/z = 1128.07; Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.12. Sulfated small molecule 2; full scan (200-1000 amu) (above) and single ion chromatogram (below) for \([\text{M} - 4 \text{ Na} + 4 \text{ HXA} + 1 \text{ HXA}]^+\) ion of observed \(m/z = 1141.75\); Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.13. Corresponding mass spectrum at 1.88 min; \([M - 4 \text{ Na} + 4 \text{ HXA} + 1 \text{ HXA}]^+\) molecular adduct ion peak of observed \(m/z = 1141.75\); Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.14. Sulfated small molecule 4; full scan (200-1000 amu) (above) and single ion chromatogram (below) for [(M – 2 Na + 2 HXA) + 1 HXA]^+ ion of observed m/z = 718.0; Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.15. Corresponding mass spectrum at 1.94 min; [(M – 2 Na + 2 HXA) + 1 HXA]^+ molecular adduct ion peak of observed m/z = 718.00; Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.16. Sulfated small molecule 5; full scan (200-1000 amu) (above) and single ion chromatogram (below) for \([(M - 1 \text{ Na} + 1 \text{ HXA}) + 1 \text{ HXA}]^+ \) ion of observed \(m/z = 781.0\); Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.17. Corresponding mass spectrum at 3.23 min; \([M - 1 \text{ Na} + 1 \text{ HXA} + 1 \text{ HXA}]^+\) molecular adduct ion peak of observed \(m/z = 781.00\); Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.18. Plot showing retention factor ($k'$) of molecule 1 (inset) in the presence of linear amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent.
Figure 3.19. Plot showing retention factor ($k'$) of molecule 2 (inset) in the presence of linear amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent.
Figure 3.20. Plot showing retention factor \((k')\) of molecule 3 (inset) in the presence of linear amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent.
Figure 3.21. Plot showing retention factor ($k'$) of molecule 4 (inset) in the presence of linear amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent.
Figure 3.22. Plot showing retention factor ($k'$) of molecule 5 (inset) in the presence of linear amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent.
Figure 3.23. Plot showing retention factor ($k'$) of molecule 1 (inset) in the presence of tertiary amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent, TPA or TPentA.
Figure 3.24. Plot showing retention factor ($k'$) of molecule 2 (inset) in the presence of tertiary amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent, TPA or TPentA.
Figure 3.25. Plot showing retention factor ($k'$) of molecule 3 (inset) in the presence of tertiary amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent, TPA or TPentA.
Figure 3.26. Plot showing retention factor ($k'$) of molecule 4 (inset) in the presence of tertiary amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent, TPA or TPentA.
Figure 3.27. Plot showing retention factor ($k'$) of molecule 5 (inset) in the presence of tertiary amines (ion-pairing agents) at concentrations ranging 5 to 40 mM. Solvent A, water containing 25 mM ‘X’ + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM ‘X’ + 0.1% v/v formic acid; cone voltage, 20 V. X = ion pairing agent, TPA or TPentA.
Figure 3.28. Slopes of retention factors ($k'$) of molecules 1-5 at 15 mM ion-pairing agent concentration (carbon atom number 4 to 9), indicating linear relationship between $k'$ and hydrophobicity of ion-pairing agent (irrespective of hydrophobic nature/surface area of retained molecule). Slope values ($m$), however, show direct proportionality with number of sulfate groups on retained molecule.
Figure 3.29. Slopes of retention factors ($k'$) of molecules 1-5 at 25 mM ion-pairing agent concentration (carbon atom number 4 to 9). Slope values ($m$) show the same properties as in case of 15 mM ion-pairing agent concentration.
Figure 3.30. $k'$ of molecule 2 in the presence of two ion-pairing agents of the same carbon atom number ($C = 9$) but different structures i.e. nonylamine, a primary amine and tripropylamine, a tertiary amine.
3.3.2. RP-IP UPLC-MS Analysis of Sucrose Octasulfate (SOS)

Introduction

Sucrose octasulfate is a small molecule comprising of a sucrose core scaffold, decorated with eight sulfate groups. Owing to its anionic nature, it has been observed to mimic interactions of cell-surface and free GAGs like chondroitin A and heparin with various ligands such as chemokine CCL complexes, fibroblast growth factor, thrombin, etc. Recognizing that GAGs are structurally complex and challenging to synthesize, focus has been shifting towards use of small molecule inhibitors such as SOS in many therapies including anti-coagulation. SOS has also been found to show anti-viral properties by inhibiting interactions of viral amino acid residues with cell-surface GAGs. The aluminum salt of SOS is a commonly prescribed anti-ulcer agent (Sucralfate). In a study by Folkman et al., the binding of sucralfate to fibroblast growth factor (FGF) and its subsequent stabilization was reported. Subsequently, Volkin et al. reported that potassium salt of SOS also bound and stabilized FGF, triggering interest in investigating water-soluble SOS (sodium and potassium salts) as a potential heparin mimic. More recently, Sarilla et al. reported SOS as competing with heparin in binding to heparin cofactor II (HCII), an allosteric inactivator of thrombin.
The structural characterization of SOS, complete with its eight sulfates was first reported in its potassium heptahydrate salt form in 1992 by Silvey GL, using $^1$H and $^{13}$C NMR. This was followed by another study of SOS structure in solution, using 2D-NMR. This report by Desai et al. compared solid state (by X-ray crystallography) and solution phase (nuclear Overhauser effect spectroscopy (NOESY)) SOS structural data. The observed conformational differences were similar to those of sucrose octaacetate. Using X-ray crystallography, SOS was found to bind to thrombin with high affinity at exosite II, with a 1:1 (SOS:BT monomer) binding stoichiometry. Results from a spectrozyme TH hydrolysis assay showed higher affinity (lower $K_d$ values) of SOS for thrombin than heparin and this was attributed to greater specificity of non-ionic interactions and solvent effects. Other spectroscopic methods, such as MS, have also been used to obtain a variety of data pertaining to interaction of SOS with proteins such as chemokines. Yu et al. used a filtration assay followed by ESI Fourier transform ion cyclotron resonance (ESI FT-ICR) MS to find that SOS bound to MCP-1 chemokine complex in 1:2 and 1:1 (MCP-1:SOS) stoichiometries and also calculated dissociation constants of these interactions using ESI FT-ICR MS and isothermal titration calorimetry.
This study aimed at establishing MS as a tool in studying protein-ligand interactions, specifically identifying inhibitors of chemokine-GAG interactions.

The binding modes of SOS with proteins are also used as a model to understand heparin-protein binding. Yang et al. crystallized aprotinin with SOS to observe 5:1 (aprotinin monomers:SOS) binding stoichiometry. Several conformations of SOS in the crystal provided an insight into the complex and variable binding of heparin with aprotinin.

Meanwhile, MS has also been pursued as an analytical tool for the characterization of SOS, and it was soon observed that SOS tended to lose some or all of its sulfates due to fragmentation in the MS source. Methods to overcome this hurdle and analyse SOS in its entirety have been developed and reported recently. In 2003, Gunay et al. published the first ESI-MS analysis of SOS using quaternary ammonium and phosphonium salts. They observed that direct infusion of SOS with 10mM tetraethylammonium hydroxide resulted in mass spectra that are devoid of sodium-SOS base peaks and contain less fragmentation. In 2006, Laremore et al. reported the use of ionic liquid matrices 1-methylimidazolium alpha-cyano-4-hydroxycinnamate and butylammonium 2,5-dihydroxybenzoate in the MALDI-TOF MS analysis of SOS and observed relatively less fragmentation. In a following report, they used bis-1,1,3,3-tetramethylguanidinium alpha-cyano-4-hydroxycinnamate ionic liquid matrix to perform MALDI-TOF MS analysis of sodium, potassium and cesium salts of SOS, and found that the cesium and rubidium salts of SOS were least susceptible to MS fragmentation. The group successfully analyzed cesium salt of chondroitin sulfate A. In 2009, Ohara et al.
derivatized several types of saccharides including SOS using pyrenemethylguanidine and obtained MALDI-MS spectra of intact molecules at high signal intensities. In a recent study, Kinoshita et al. published the CE-UV analysis of sulfates after hydrolyzing them off sucrose octasulfate using HCl. This is a unique way of obtaining sulfate content of di- and oligo- saccharides.

In this study, our aim was to eliminate the need for complex steps or reagents and use a simple one-step ion-pairing UPLC-MS method to successfully retain and analyze SOS with all the eight sulfates intact. This method also aims to separate and identify any impurities. From here on, SOS will refer to sodium salt of SOS only.

3.3.2.1. Methods and experimental conditions

3.3.2.2. Materials

Sucrose octasulfate was purchased from Biomol International (now Enzo Life Sciences International, Inc., Plymouth Meeting, PA) and stored in -80°C, n-pentylamine (PTA), of the highest purity was purchased from Fisher Scientific (Fair Lawn, NJ). Solvents such as optima LC-MS grade acetonitrile and methanol were from Sigma (St. Louis, MO). Optima LC-MS grade formic acid was purchased from Fisher Scientific (Fair Lawn, NJ) and Nerl High-purity water from Thermo Fisher Scientific (Middletown, VA).

3.3.2.3. RP-IP UPLC-MS equipment and experimental conditions

Refer to section 2.2.2., in chapter 2.
3.3.2.4. Results and Discussion

UPLC-MS of SOS in the absence of IPA resulted in molecular ion peak co eluting with solvent in 0.26 minutes, \( k' < 1 \) (Figure 3.32). The corresponding mass spectrum (Figure 3.33) shows the presence of SOS-Na\(^+\) adduct ion; \([M + 1 Na^+]^+, m/z = 1180.5\), at a peak intensity of \(2.05 \times 10^6\). However, one should note the significant amount of noise signals, also of \(e^6\) intensity range, generated from sulfate loss peaks. Peaks of \(m/z\) equal to 1078.7, 976.9, 874.8, 772.8, 672.6 and 569.3 seen in Figure 3.33, represent SOS-Na\(^+\) ions formed after successive losses of one to six sodium sulfonates (NaSO\(_3\), mass = 102.95).

In the presence of 25 mM PTA, SOS was easily retained and characterized using reversed-phase ion-pairing UPLC-MS. In Figure 3.34, single ion chromatogram for SOS molecular adduct ion, \([SOS – 8 Na + 8 PTA] + 1 PTA\]^+\), \(m/z = 1768.7\), a well retained sharp peak of \(2.9 \times 10^7\) signal intensity, is observed at 2.10 minutes, i.e. \(k' \geq 7\).

Experiments were performed using same UPLC gradient and MS conditions discussed in previous chapters, except for cone voltage. Cone voltages ranging from 10 to 100 V were tested for optimization of MS method (Figure 3.36).

At 100 V, PTA adduct ion was observed, but at a very low intensity (\(m/z = 1767.7; 5.5 \times 10^5\)). This was because of extensive break down of SOS at the MS source, especially sulfonate group losses. This is similar to the chromatogram obtained in the absence of ion-pairing agent, where sodium adduct ion can be identified amongst a cluster of other ion peaks which maybe noise and fragment ions, making it hard to
identify the peak of interest, as it elutes along with the solvent peak. This can make it impossible to interpret data if the analyte mass is unknown.

At cone voltages between 50 to 100 V, peaks of $m/z$ equal to 1600.5, 1432.4, and 1264.3, corresponding to one, two and three sulfonate group-PTA complex losses respectively are observed. As we further reduce the cone voltage, fragmentation drastically lowers down i.e. at 20 and 10 V, only singly and doubly charged SOS-PTA ions peaks are present.

Lowering the cone voltage to 20 V in the ion-pairing method for SOS resulted in a clean spectrum with no fragmentation (see Figure 3.35). The molecular adduct ion was obtained as a PTA adduct peak of $m/z = 1768.7$ (at $1.6 \times 10^7$) but the base peak, however, was the doubly charged adduct ion, $[(SOS – 8 Na + 8 PTA) + 2 PTA]^{2+}$, $m/z = 928.5$ (at $4.0 \times 10^7$). An additional peak, $m/z = 1208.4$, was observed in the spectrum along with the singly and doubly charged molecular adduct ion peaks. This peak could be triply charged SOS trimer, after loss of six sulfates, $[(3 SOS – 20 Na^+ + 20 PTA – 6 PTA-SO_3 + 6 H^+) + 3 PTA]^{3+}$, $m/z = (3625.86)/3 = 1208.62$ (ppm error $\leq 0.2$).

At 10 V, there was no fragmentation but the intensities of the peaks were half that at 20 V i.e. singly charged molecular adduct ion, $1768.2$ at $8.1 \times 10^6$ and doubly charged molecular adduct ion, $928.2$ at $2.6 \times 10^7$ intensities. Hence 20 V was chosen as the optimal cone voltage for RP-IP UPLC-MS characterization of sodium salt of sucrose octasulfate. On basis of results from the RP-IP UPLC-MS analysis of molecules 1 to 5, 25 mM HXA is also expected to be a suitable ion-pairing agent for the analysis of SOS, possibly retaining SOS better than PTA, on the column.
Figure 3.32. SIR chromatogram for SOS sodium adduct ion in the absence of ion-pairing agents, mass = 1180.6, Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 100 V. No retention observed as SOS elutes with the solvent peak at 0.26 minutes.
Figure 3.33. Corresponding mass spectrum at 0.26 minutes. Spectrum shows SOS sodium adduct ion peak, $m/z = 1180.5$ as well as several extraneous peaks corresponding to ions resulting from sulfate losses due to fragmentation. Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 100 V.
Figure 3.34. SIR chromatogram for SOS-PTA adduct ion, m/z = 1768.7. Solvent A, water containing 25 mM PTA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM PTA + 0.1% v/v formic acid; cone voltage, 20 V. The single peak at 2.10 minutes has two other closely eluting peaks which seem to be stereoisomeric peaks since all 3 peaks gave the same mass spectra.
Figure 3.35. Mass spectrum for above chromatogram at 2.10 minutes. Spectrum shows SOS-PTA adduct ion peak, $m/z = 1768.7$ as well as doubly charged SOS-PTA ion peak, $m/z = 928.5$.

Solvent A, water containing 25 mM PTA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM PTA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.36. Ion pairing with 25 mM PTA. Mass spectra for SOS-PTA adduct ion at different cone voltages. From top: 100 V; 50 V; 40 V; 20 V; 10 V. Solvent A, water containing 25 mM PTA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM PTA + 0.1% v/v formic acid.
3.3.3. RP-IP UPLC-MS analysis of flavanoid dimers 6 and 7

In order to test our ion-pairing reversed-phase UPLC-MS method on higher molecular weight molecules (MW > 1000), two flavanoid dimer molecules sulfated in our lab were used, which for sake of convenience will be referred to as 6 and 7. The method used for the molecules described in the previous chapters was followed and since HXA proved to be better retaining agent than PTA, 25 mM HXA was chosen as the ion-pairing agent for the following experiments.

Figure 3.37. Flavanoid dimers 6 and 7 (clockwise from left), m/z = 1461.64 and 1257.76 respectively

3.3.3.1. Methods and experimental conditions
3.3.3.2. Materials

Molecules 6 and 7 (synthesized by the Desai lab and stored in -80°C), n-hexylamine (HXA) of the highest purity was purchased from Fisher Scientific (Fair Lawn, NJ).

3.3.3.3. RP-IP UPLC-MS equipment and experimental conditions

Ultraperformance liquid chromatography (UPLC) was used in conjunction with UV spectrophotometry and electrospray ionization-mass spectrometry (ESI-MS) in order to retain and identify CS. Waters Acquity H-class UPLC system equipped with Acquity photodiode array detector and triple quadrupole mass spectrometer (TQD-MS) was used to perform all experiments.

The eluent from the UPLC was directly introduced into the UV spectrophotometer, followed by the TQD-MS. UV wavelength was set at 190–400 nm range, with a resolution of 1.2 nm and the sampling rate was set at 20 points per second. All mass spectrometry was carried out in positive mode and for MS tuning, capillary voltage was set at 4 kV, cone voltage at 20 V, desolvation temperature at 350°C and gas flow at 650 L/hr. UPLC-MS method consisted of scans for m/z ranges of 200-2048 with scan time of 0.25 seconds. Selected Ion Recording (SIR) with a dwell time of 0.025 seconds was also performed for masses corresponding to singly charged ‘compound-ion pairing agent’ molecular adduct ion; \( m/z = [M - x \text{ Na} + (x + 1) \text{ HXA}]^+ \), where ‘x’= number of sulfates.

3.3.3.4. Results and Discussion
Similar to all previously described small molecules, both 6 and 7 show early elution times i.e., at 0.30 minutes after the solvent peaks (0.22 minutes) under normal UPLC-MS conditions (Figure 3.38) hence, \( k' \leq 0.4 \). Despite the advanced UPLC system and high pressures, these highly water-soluble compounds are not retained by any significant degree. Also, in the MS, due to the thermolabile nature of sulfonate groups, they break down into smaller fragments by losing their sulfonates and also undergo inter and intra-ring cleavages (Figure 3.39), resulting in a mass spectrum containing too many peaks, with no useful information.

To characterize 6 and 7, 25 mM HXA was used as the ion-pairing agent, since these molecules are larger and a longer chain ion-pairing agent ensures greater retention than a shorter chain IPA of the same concentration. Figures 3.40 to 3.43 show that molecules 6 and 7, which under normal conditions eluted at 0.3 minutes, were retained till 2.68 and 2.59 minutes respectively, in the presence of 25 mM HXA, whilst no changes in gradient conditions. Solvent peaks are observed at 0.21 and 0.27 minutes during RPIP UPLC-MS of 6 and 7, respectively, hence \( k' \) of 6 = 11.7 and \( k' \) of 7 = 8.59. Peaks eluting early at 2.48 and 2.56 minutes on the UV chromatogram of 6 (Figure 3.40), represent partially sulfated impurities resulting from incomplete synthesis and/ or degradation over time of storage. As these impurities are less hydrophilic, they ion-pair with fewer HXA ions, hence, elute slightly earlier than the fully sulfated molecule. Separation of these peaks depends on a number of factors, such as; gradient, strength of organic mobile phase, structure and alkyl chain length of IPA etc.
Mass spectra (Figures 3.41 and 3.43) show the presence of molecular ions as doubly charged HXA adduct ions. Molecular- HXA adduct ion of 6; \([\text{M} – 8 \text{Na} + 8 \text{HXA}]^{2+}\), \(m/z = 1149.94\) (ppm error < 0.5) is observed at an intensity of 2.7 \(e^6\).

Similarly, for 7; \([(\text{M} – 8 \text{Na} + 8 \text{HXA}) + 1 \text{ HXA}]^{1+}\), \(m/z = 1834.55\) (ppm error < 0.2), recorded at an intensity of 5.0 \(e^6\). Also seen in the spectrum is doubly charged molecular adduct ion, \([(\text{M} – 6 \text{Na} + 6 \text{HXA}) + 2 \text{ HXA}]^{2+}\), \(m/z = 968.92\) (ppm error < 0.5).

Confirmation of charge of a molecular adduct ion peak can be made by examining the consecutive sulfonate loss peak. For example, Figure 3.41 shows the presence of a low intense peak (1.3 \(e^5\)) of \(m/z = 1652.5\). This peak results from loss of a single sulfonate-HXA group from singly charged molecular adduct ion i.e. 1834.55 – (80 + 102.19 + 1H) = 1652.5 (ppm error ≤ 0.8). Addition of hydrogen balances out the negatively charged oxygen that is left behind after loss of sulfonate. On the other hand, doubly charged molecular adduct ion peak; \(m/z = 968.92\) loses its sulfonate-HXA, i.e. \(m/z = [1936.74 – (80 + 102.19 + 1H)]/2 = 878.64\) (ppm error < 1). Thus, the mass difference between doubly charged molecular adduct ion and its single sulfonate loss ion, thus, is 90.28 (968.92 – 878.64). This is approximately equal to mass of (sulfonate-HXA – 1 H)/2 = 181.19/2 = 90.59.

Therefore, charge of molecular ion-IPA peak obtained using our RPIP UPLC MS method can be determined by this simple formula;

\[
\text{Charge, } n = \frac{\text{mass of (sulfonate-IPA – 1H)}}{\text{(mass difference between two consecutive } m/z \text{ peaks)}}
\]
This simple calculation can help in identifying MS peaks by correlating sulfonate losses with molecular adduct ion charges.

The above results show the successful use of our RPIP UPLC-MS method in the characterization of sulfated flavanoid dimers and determination of charge by using sulfonate loss information. In conclusion, this method has proven robust enough to be applied to a wide range of compounds differing in scaffold type, molecular weights, hydrophobic surface areas and number of sulfates.
Figure 3.38. UV chromatogram (above) and mass chromatogram (below) for molecule 6 in the absence of ion-pairing agents, Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 100 V. No retention observed as SOS elutes with the solvent peak at 0.26 minutes.
Figure 3.39. Corresponding mass spectrum of peak at 0.27 minutes, numerous peaks at noise level and resulting out of fragmentation, making it impossible to identify the molecular ion peak.
Figure 3.40. UV spectrum, total ion count mass chromatogram and single ion count chromatogram respectively (from top) of 6 in the presence of 25 mM HXA. UV peaks at 2.49 and 2.56 minutes indicate degradation products (devoid of some sulfates) of 6. Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.41. Mass spectrum of total ion count chromatogram of 6 at 2.7 minutes, containing base peak of $m/z = 1149.9$ which corresponds to doubly charged adduct ion $[(M + 8 \text{ HXA}) + 2 \text{ HXA}]^{2+}$. Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.42. UV spectrum, mass chromatogram and total ion count chromatogram respectively (from top) of 7 in the presence of 25 mM HXA. UV peaks at 2.70 and 2.81 minutes indicate degradation products (devoid of some sulfates) of 7. Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 3.43. Mass spectrum of total ion count chromatogram of 7 at 2.6 minutes, containing base peak of $m/z = 1834.5$ which corresponds to singly charged adduct ion $[(M + 6 \text{ HXA}) + 2 \text{ HXA}]^{+}$. Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; cone voltage, 20 V.
CHAPTER 4

Mass Spectrometric Fingerprinting Of Sulfated Small Molecules

In 1919, based on J. J. Thompson’s discovery of neon isotopes, his student, Francis William Aston, reported the construction of the first mass spectrograph,\textsuperscript{133} for which he received the Nobel Prize in chemistry in 1921. While mass spectrometry has ever since been used to obtain the mass of a molecule, the introduction of another stage of mass analysis, in 1968, helped further obtain detailed structural information by means of “collision-induced dissociation”\textsuperscript{134}. This came to be known as tandem MS or MS/MS. Fingerprints of analytes can be developed using tandem MS, which in a nutshell, can be explained as the fragmentation of ions (formed in the MS source) in a collision cell containing an inert gas like helium or argon, generating unique daughter ions that are subsequently detected by a second mass analyzer that is present after the collision cell. Tandem MS or MS/MS is a popular and versatile structural tool with applications ranging from analyzing the yeast proteome\textsuperscript{135} to fingerprinting different kinds of beer\textsuperscript{136}. In addition to tandem MS, MS alone can also be used for fingerprinting purposes, making use of a phenomenon called “up-front collision-induced dissociation” (also called in-source fragmentation). This method is convenient and does not require a second mass analyzer, thus also named ‘the poor man’s MS/MS’.\textsuperscript{137}

When parameters such as MS capillary voltage and cone voltage are suitably controlled, two regions that favor fragmentation are found; one being the region between the capillary and the skimmer cone, and the second is between the skimmer cone and the
quadrupole (or the extraction cone, when it is present). Due to voltage differences, the ions in these regions accelerate and as a result begin to collide with each other and surrounding nitrogen gas to give fragment ions (Figure 4.1). This is known as up-front collision-induced dissociation (up-front CID).

Up-front CID occurs during most analyses, especially when the MS conditions are too harsh for the analyte. Bond breaking may give a good number of peaks in the mass spectrum that may provide useful information or may also result in information loss. This phenomenon can be used to our advantage by controlling certain parameters. One such important parameter is the cone voltage or the skimmer cone voltage. By maintaining MS source conditions ambient for controlled up-front CID; we have been able to develop unique fingerprints for all of the compounds that we tested using RPIP-UPLC MS.

Figure 4.1 Schematic of up-front CID, V, velocity; N, nozzle; S, skimmer cone; Q, quadrupole.137
4.2 Experimental methods

4.2.1. Materials

Molecules 1 to 7 (synthesized by the Desai lab and stored in - 80°C), Ion-pairing agent, n-hexylamine (HXA) of the highest purity was purchased from Fisher Scientific (Fair Lawn, NJ).

4.2.2. RP-IP UPLC-MS equipment and experimental conditions

Refer to chapter 3, section 3.3.3.3.

4.2.3 Results and Discussion

In normal UPLC-MS conditions with no ion-pairing agents, sulfate groups of analytes are paired with sodium ions or protons and are observed to be lost easily and in a random fashion (Refer to chapters two and three).

Ion-pairing chromatography of sulfated molecules in presence of 25mM HXA was performed and MS data was collected at cone voltages 20, 40 and 60 V. In case of CS, at 20 V, there was no sulfonate loss observed and its intact molecular adduct ion, M₁; [(M – 5 Na + 5 HXA) + 1 HXA] \(^{1+}\), \( m/z = 1297.59 \), was detected and recorded by the mass spectrometer (Figure 4.3). When the cone voltage was increased to 40 V, the obtained mass spectrum showed prominent peaks, M₁ and M₂; \([M₁]^{1+} - (1 \text{ HXA}^+ - \text{SO}_3^- +1 \text{ H}^+)\), \( m/z = 1116.52 \), corresponding to the CS molecular adduct ion and CS with one sulfonate-HXA pair loss respectively. Similarly at 60 V, further fragmentation up to loss of four sulfonate groups was observed (Figure 4.3 ; Table 1). Similar losses were also
observed in case of RPIP UPLC-MS of molecules 1 to 7, when ion-paired with 25 mM HXA (Figures 4.4 to 4.9; Tables 2 to 7). Sulfonate losses in the presence of ion-pairing agent were observed to be systematic, following a reproducible pattern. By implementing this simple yet unique method, we were able to fingerprint our sulfated small molecules (Figures 4.3 to 4.9, Tables 1 to 7), and also obtain the number of minimum sulfate groups on the molecule by examining the number of sulfonate losses. A loss of four sulfonates, as seen in case of molecule 3 at 60 V (Figure 4.3), guarantees the presence of at least four sulfates in the molecule.

In addition, reproducible sulfonate loss patterns at a particular cone voltage serve as an important mathematical tool in understanding complex chromatograms of larger molecules such as sulfated polymers. As we note from all the MS spectra (Figures 4.3 to 4.9) in this chapter and the previous chapter, a sulfonate loss spectrum always shows sets of peaks differing by a definite $m/z$ value. This value could correspond to $m/z$ of a single sulfonate group (mass = 79.96) or (sulfonate group-IPA)/n; where $n$ = charge carried by the ion. Thus, by calculating the difference between the $m/z$ values of two consecutive MS peaks, one can determine the charge of an ion peak.

With increments in cone voltage to 20 V to 60 V, we observed that sulfated molecules gradually lost their sulfate groups due to up-front CID and these losses with increasing cone voltages were found to be highly reproducible when ion-pairing chromatography was used as opposed to normal UPLC-MS where up-front CID was random and occurred at lower cone voltages. It can thus be understood that ion-pairing
agents such as HXA are sulfate-group protecting and break off the molecule, ion-paired with sulfonate groups, when cone voltage is increased.

Further up-front CID studies on different aromatic and non-aromatic sulfated molecules by modifying different parameters such as cone and capillary voltage, may help identify the positions of sulfate groups on the core scaffold. This information can help sequence unknown sulfated GAGs of medical importance and perhaps, understand the way in which sulfate groups assist in the binding of GAGs to proteins.

Figure 4.2. Singly-charged CS (3) molecular adduct ion droplet, [(M – 5 Na + 5 HXA) + 1 HXA] \(^{1+}\) = 1297.59; formed in the MS source, in the presence of 25 mM HXA. The circled regions indicate labile sulfonate groups that break off the molecular adduct ion during up-front CID when MS parameters such as cone voltage are increased.
Figure 4.3. MS fingerprint of CS at cone voltages 20 V, 40 V and 60 V respectively (from top). Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; MS capillary voltage, 4 kV; desolvation temperature, 350°C and gas flow, 650 L/hr.
Table 1. In-source CID losses demonstrated by CS, ion-paired with 25 mM HXA at cone voltages 30 V, 40 V and 60 V, capillary voltage; 4 kV, desolvation temperature; 350°C and gas flow; 650 L/hr.

<table>
<thead>
<tr>
<th>Cone voltage (V)</th>
<th>Peak</th>
<th>m/z formula</th>
<th>Calculated m/z</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>M₁</td>
<td>[(M – 5 Na + 5 HXA) + 1 HXA](^{1+})</td>
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<td>1298.45</td>
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<td></td>
<td>[(M – 5 Na + 5 HXA) + 2 HXA](^{2+})</td>
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<td>40</td>
<td>M₁</td>
<td>[(M – 5 Na + 5 HXA) + 1 HXA](^{1+})</td>
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<td>1297.17</td>
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<td></td>
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<td>[M₁(^{1+}) – 1 HXA(^{+}) - SO₃(^{-}) + 1 H(^{+})]</td>
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<td>1116.43</td>
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<tr>
<td>60</td>
<td>M₁</td>
<td>[(M – 5 Na + 5 HXA) + 1 HXA](^{1+})</td>
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<td>1297.09</td>
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<td>[M₁(^{1+}) – 1 HXA(^{+}) - SO₃(^{-}) + 1 H(^{+})]</td>
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<td>[M₁(^{1+}) – 3 HXA(^{+}) - 2 SO₃(^{-}) + 3 H(^{+})]</td>
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<td></td>
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<td>754.36</td>
<td>753.77</td>
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Figure 4.4. MS fingerprint of 1 at cone voltages 20 V, 40 V and 60 V respectively (from top).
Table 2. In-source CID losses demonstrated by molecule 1, ion-paired with 25 mM HXA, at cone voltages 30 V, 40 V and 60 V, capillary voltage; 4 kV, desolation temperature; 350°C and gas flow; 650 L/hr.

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<th>Cone voltage (V)</th>
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<th>$m/z$ formula</th>
<th>Calculated $m/z$</th>
<th>Observed $m/z$</th>
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</thead>
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<td>20</td>
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<td>1128.07</td>
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<td>945.81</td>
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<td>1127.11</td>
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<td></td>
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<td>945.97</td>
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<td>60</td>
<td>M$_3$</td>
<td>M$_1$$^{+}$ – 2 HXA$^{+}$ - SO$_3^{-}$ + 2 H$^{+}$</td>
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<td>M$_4$</td>
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<td>764.97</td>
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Figure 4.5. MS fingerprint of 2 at cone voltages 20 V, 40 V and 60 V respectively (from top).
Table 3. In-source CID losses demonstrated by molecule 2, ion-paired with 25 mM HXA, at cone voltages 30 V, 40 V and 60 V, capillary voltage; 4 kV, desolvation temperature; 350°C and gas flow; 650 L/hr.

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<th>Calculated m/z</th>
<th>Observed m/z</th>
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<td>20</td>
<td>M₁</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA]⁺</td>
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<td>1141.75</td>
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<td>40</td>
<td>M₁</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA]⁺</td>
<td>1141.55</td>
<td>1141.79</td>
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<td>M₂</td>
<td>[M₁⁻]⁻ – 1 HXA⁺ - SO₃⁻ + 1 H⁺</td>
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<td>960.57</td>
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<tr>
<td>60</td>
<td>M₃</td>
<td>[M₁⁻]⁻ – 2 HXA⁺ - SO₃⁻ + 2 H⁺</td>
<td>859.35</td>
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<td>M₄</td>
<td>[M₁⁻]⁻ – 2 HXA⁺ - 2SO₃⁻ + 2 H⁺</td>
<td>779.39</td>
<td>778.85</td>
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<td></td>
<td>M₅</td>
<td>[M₁⁻]⁻ – 3 HXA⁺ - 2SO₃⁻ + 3 H⁺</td>
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<td>677.75</td>
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<td>M₆</td>
<td>[M₁⁻]⁻ – 3 HXA⁺ - 3SO₃⁻ + 3 H⁺</td>
<td>598.32</td>
<td>597.81</td>
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Figure 4.6. MS fingerprint of 4 at cone voltages 20 V, 40 V and 60 V respectively (from top).
Table 4. In-source CID losses demonstrated by molecule 4, ion-paired with 25 mM HXA, at cone voltages 20 V, 40 V and 60 V, capillary voltage; 4 kV, desolvation temperature; 350°C and gas flow; 650 L/hr.

<table>
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<th>Cone voltage (V)</th>
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<th>m/z formula</th>
<th>Calculated m/z</th>
<th>Observed m/z</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>M₁</td>
<td>[(M – 2 Na + 2 HXA) + 1 HXA]¹⁺</td>
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<td>718.00</td>
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<td></td>
<td>M₁</td>
<td>[(M – 2 Na + 2 HXA) + 1 HXA]¹⁺</td>
<td>718.35</td>
<td>718.06</td>
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<td></td>
<td>M₂</td>
<td>[M₁]¹⁺ – 1 HXA⁺ + 1 H⁺</td>
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<td>616.83</td>
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<td>M₃</td>
<td>[M₁]¹⁺ – 1 HXA⁺ – 1 SO₃⁻ + 1 H⁺</td>
<td>537.27</td>
<td>536.95</td>
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<td></td>
<td>M₄</td>
<td>[M₁]¹⁺ – 2 HXA⁺ – 2 SO₃⁻ + 2 H⁺</td>
<td>435.15</td>
<td>435.75</td>
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<tr>
<td>40</td>
<td>M₁</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA]¹⁺</td>
<td>718.35</td>
<td>---</td>
</tr>
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<td></td>
<td>M₂</td>
<td>[M₁]¹⁺ – 1 HXA⁺ – SO₃⁻ + 1 H⁺</td>
<td>617.23</td>
<td>616.95</td>
</tr>
<tr>
<td></td>
<td>M₃</td>
<td>[M₁]¹⁺ – 2 HXA⁺ – SO₃⁻ + 2 H⁺</td>
<td>537.27</td>
<td>536.79</td>
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<tr>
<td></td>
<td>M₄</td>
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<td>435.78</td>
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<tr>
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<td>M₁</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA]¹⁺</td>
<td>718.35</td>
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</tr>
<tr>
<td></td>
<td>M₂</td>
<td>[M₁]¹⁺ – 1 HXA⁺ – SO₃⁻ + 1 H⁺</td>
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<td>616.95</td>
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<tr>
<td></td>
<td>M₃</td>
<td>[M₁]¹⁺ – 2 HXA⁺ – SO₃⁻ + 2 H⁺</td>
<td>537.27</td>
<td>536.79</td>
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<td></td>
<td>M₄</td>
<td>[M₁]¹⁺ – 2 HXA⁺ – 2 SO₃⁻ + 2 H⁺</td>
<td>435.15</td>
<td>435.78</td>
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</table>
Figure 4.7. MS fingerprint of 5 at cone voltages 20 V, 40 V and 60 V respectively (from top).
Table 5. In-source CID losses demonstrated by molecule 5, ion-paired with 25 mM HXA, at cone voltages 20 V, 40 V and 60 V, capillary voltage; 4 kV, desolvation temperature; 350°C and gas flow; 650 L/hr.

<table>
<thead>
<tr>
<th>Cone voltage (V)</th>
<th>Peak</th>
<th>m/z formula</th>
<th>Calculated m/z</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>M₁</td>
<td>[(M – 1 Na + 1 HXA) + 1 HXA] (^{1+})</td>
<td>781.36</td>
<td>781.00</td>
</tr>
<tr>
<td></td>
<td>M₂</td>
<td>[M₁] (^{1+}) – (1 HXA(^{+}) + 1 H(^{+}))</td>
<td>680.24</td>
<td>679.86</td>
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<td></td>
<td>M₃</td>
<td>[M₁] (^{1+}) – 1 HXA(^{+}) - 1 SO₃(^{-}) + 1 H(^{+})</td>
<td>600.28</td>
<td>600.06</td>
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<td>40</td>
<td>M₁</td>
<td>[(M – 1 Na + 1 HXA) + 1 HXA] (^{1+})</td>
<td>781.36</td>
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<td>[M₁] (^{1+}) – (1 HXA(^{+}) + 1 H(^{+}))</td>
<td>680.24</td>
<td>679.86</td>
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<td></td>
<td>M₃</td>
<td>[M₁] (^{1+}) – 1 HXA(^{+}) - 1 SO₃(^{-}) + 1 H(^{+})</td>
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<td>599.90</td>
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<td>M₁</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA] (^{1+})</td>
<td>781.36</td>
<td>780.94</td>
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Figure 4.8. MS fingerprint of 6 at cone voltages 20 V, 40 V and 60 V respectively (from top).
Figure 4.9. MS fingerprint of 7 at cone voltages 20 V, 40 V and 60 V respectively (from top).
6, [(M – 8 Na + 8 HXA) + 2 HXA]^{2+}, m/z = 1149.50

7, [(M – 6 Na + 6 HXA) + 1 HXA]^{1+}, m/z = 1834.72
Table 6. In-source CID losses demonstrated by molecule 6, ion-paired with 25 mM HXA, at cone voltages 20 V, 40 V and 60 V, capillary voltage; 4 kV, desolvation temperature; 350°C and gas flow; 650 L/hr.

<table>
<thead>
<tr>
<th>Cone voltage (V)</th>
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<th>Observed m/z</th>
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<td>20</td>
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<td>[(M – 8 Na + 8 HXA) + 2 HXA]^{2+}</td>
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<td>1149.51</td>
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<td>M₂</td>
<td>[(M – 8 Na + 8 HXA) + 2 HXA]^{2+}</td>
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<td>[(M – 8 Na + 8 HXA) + 1 HXA]^{1+}</td>
<td>1149.50</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>M₂</td>
<td>[M₁]^{2+} – 1 HXA⁺ - 1 SO₃⁻ + 1 H⁺</td>
<td>1058.94</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>M₃</td>
<td>[M₁]^{2+} – 2 HXA⁺ - 1 SO₃⁻ + 2 H⁺</td>
<td>1008.37</td>
<td>1009.00</td>
</tr>
<tr>
<td></td>
<td>M₄</td>
<td>[M₁]^{2+} – 2 HXA⁺ - 2 SO₃⁻ + 2 H⁺</td>
<td>968.37</td>
<td>968.051</td>
</tr>
<tr>
<td></td>
<td>M₅</td>
<td>[M₁]^{2+} – 3 HXA⁺ - 2 SO₃⁻ + 3 H⁺</td>
<td>917.30</td>
<td>918.31</td>
</tr>
<tr>
<td></td>
<td>M₆</td>
<td>[M₁]^{2+} – 3 HXA⁺ - 3 SO₃⁻ + 3 H⁺</td>
<td>877.80</td>
<td>877.67</td>
</tr>
<tr>
<td></td>
<td>M₇</td>
<td>[M₁]^{2+} – 4 HXA⁺ - 3 SO₃⁻ + 4 H⁺</td>
<td>827.23</td>
<td>826.69</td>
</tr>
<tr>
<td></td>
<td>M₈</td>
<td>[M₁]^{2+} – 5 HXA⁺ - 4 SO₃⁻ + 4 H⁺</td>
<td>736.12</td>
<td>736.21</td>
</tr>
</tbody>
</table>
Table 7. In-source CID losses demonstrated by molecule 7, ion-paired with 25 mM HXA, at cone voltages 20 V, 40 V and 60 V, capillary voltage; 4 kV, desolvation temperature; 350°C and gas flow ; 650 L/hr.

<table>
<thead>
<tr>
<th>Cone Voltage (V)</th>
<th>Peak</th>
<th>m/z formula</th>
<th>Calculated m/z</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>M₁</td>
<td>[(M – 6 Na + 6 HXA) + 1 HXA] (^{1+})</td>
<td>1834.72</td>
<td>1834.55</td>
</tr>
<tr>
<td></td>
<td>M₁'</td>
<td>[(M – 6 Na + 6 HXA) + 2 HXA] (^{2+})</td>
<td>968.43</td>
<td>968.92</td>
</tr>
<tr>
<td>40</td>
<td>M₁</td>
<td>[(M – 6 Na + 6 HXA) + 1 HXA] (^{1+})</td>
<td>1834.72</td>
<td>1833.12</td>
</tr>
<tr>
<td></td>
<td>M₂</td>
<td>[M₁] (^{1+}) – 1 HXA(^+) – 1 SO(_3)^{-} + 1 H(^+)</td>
<td>1653.59</td>
<td>1653.54</td>
</tr>
<tr>
<td></td>
<td>M₃</td>
<td>[M₁] (^{1+}) – 2 HXA(^+) – 2 SO(_3)^{-} + 2 H(^+)</td>
<td>1472.46</td>
<td>1471.53</td>
</tr>
<tr>
<td></td>
<td>M₄</td>
<td>[M₁] (^{1+}) – 3 HXA(^+) – 3 SO(_3)^{-} + 3 H(^+)</td>
<td>1291.33</td>
<td>1290.56</td>
</tr>
<tr>
<td></td>
<td>M₁'</td>
<td>[(M – 6 Na + 6 HXA) + 2 HXA] (^{2+})</td>
<td>968.43</td>
<td>968.88</td>
</tr>
<tr>
<td></td>
<td>M₂'</td>
<td>[M₁] (^{2+}) – 1 HXA(^+) – 1 SO(_3)^{-} + 1 H(^+)</td>
<td>877.86</td>
<td>877.71</td>
</tr>
<tr>
<td></td>
<td>M₃'</td>
<td>[M₁] (^{2+}) – 2 HXA(^+) – 2 SO(_3)^{-} + 2 H(^+)</td>
<td>787.29</td>
<td>786.77</td>
</tr>
<tr>
<td>60</td>
<td>M₁</td>
<td>[(M – 6 Na + 6 HXA) + 1 HXA] (^{1+})</td>
<td>1834.72</td>
<td>1834.14</td>
</tr>
<tr>
<td></td>
<td>M₂</td>
<td>[M₁] (^{1+}) – 1 HXA(^+) – 1 SO(_3)^{-} + 2 H(^+)</td>
<td>1653.59</td>
<td>1653.65</td>
</tr>
<tr>
<td></td>
<td>M₃</td>
<td>[M₁] (^{1+}) – 2 HXA(^+) – 2 SO(_3)^{-} + 2 H(^+)</td>
<td>1472.46</td>
<td>1471.78</td>
</tr>
<tr>
<td></td>
<td>M₄</td>
<td>[M₁] (^{1+}) – 3 HXA(^+) – 3 SO(_3)^{-} + 3 H(^+)</td>
<td>1291.33</td>
<td>1290.49</td>
</tr>
<tr>
<td></td>
<td>M₅</td>
<td>[M₁] (^{1+}) – 4 HXA(^+) – 4 SO(_3)^{-} + 4 H(^+)</td>
<td>1110.20</td>
<td>1108.64</td>
</tr>
<tr>
<td></td>
<td>M₆</td>
<td>[M₁] (^{1+}) – 5 HXA(^+) – 5 SO(_3)^{-} + 5 H(^+)</td>
<td>929.07</td>
<td>927.36</td>
</tr>
</tbody>
</table>
4.3 Fingerprints of positional isomers

So far our RP-IP UPLC-MS method proved to be successful in analysis and fingerprinting of sulfated small molecules of various scaffolds and degree of sulfation. Our next step aimed at identifying any differences in fragmentation patterns amongst small molecules of the same molecular weight, scaffold and degree of sulfation but different in positions of decorating sulfate groups i.e. positional isomers. In order to proceed with this study, hydroxyl derivatives of three flavonoid small molecules (Figure 4.10) were purchased and sulfated in our lab.

Figure 4.10. Clockwise from left: 3, 5, 7, 4’ - Tetrahydroxyflavone (8); 3, 7, 3’, 4’ - Tetrahydroxyflavone (9); 5, 7, 3’, 4’ - Tetrahydroxyflavone (10)
Synthesis:

50 mg of polyphenol flavanoid was taken in a microwavable tube and to it were added 24 equivalents (6 eq. per –OH) of triethylamine sulfur trioxide complex (Et₃N:SO₃), 4 equivalents (1 eq. per –OH) of triethylamine (Et₃N) and 1 ml of acetonitrile (MeCN). This mixture was sealed and microwaved for two hours at 100°C (CEM Discover synthesizer, Cary, NC). After microwaving, the mixture was allowed to cool before being tested for completion of sulfation by performing thin-layer chromatography (TLC). Silica plates were used as the stationary phase and solvent contained 30% methanol in dichloromethane (DCM). The mixture was divided into several pools which were spotted onto the TLC plate using a capillary, to examine the progress of the sulfation reaction. Rotary evaporator (Rotavap) was used to evaporate excess methanol and DCM. After evaporation, DCM was added in sufficient volumes, followed by three spoons of silica and the mixture was put back into the rotavap, till dry. Finally, purification step was carried out by performing flash chromatography, using gradient increase in % methanol/DCM mixture. Purified mixture was loaded onto an SP Sephadex C25 column to exchange TEA with sodium (Na). Fractions of the eluate were collected and lyophilized for 2 to 3 days, until powder was obtained.

Figure 4.11. Schematic of sulfation reaction; precursor, 9, is sulfated to persulfated product, 9s.
NMR structure elucidation:

$^{1}$H and $^{13}$C spectroscopy were used to characterize compounds 8$s$, 9$s$ and 10$s$, dissolved in D$_2$O and DMSO (see Figures 4.19 to 4.25). Experiments were performed using Bruker 400 MHz NMR spectrometer, reporting chemical shifts (in ppm).

**Sulfated compound 8 (8s).** $^{1}$H-NMR (D$_2$O, 400 MHz): 8.11 (m, 2 H), 7.46 (m, 2 H), 7.12 (d, 1 H), 6.8 (d, 1 H), $^{1}$H-NMR (DMSO, 400 MHz): 12.36 (s, 1 H), 8.13 (m, 2H), 7.21 (m, 2H), 6.91 (d, 1H), 6.54 (d, 1H), $^{13}$C-NMR (DMSO, 100 MHz): 178.01, 160.21, 159.62, 156.57, 155.60, 133.22, 129.74, 124.42, 119.24, 106.11, 101.90, 97.52, 52.82.

**Sulfated compound 9 (9s).** $^{1}$H-NMR (DMSO, 400 MHz): 8.19 (d, 1 H), 8.07 (m, 1 H), 7.99 (d, 1 H), 7.65 (d, 1 H), 7.46 (m, 1 H), 7.23 (m, 1 H), $^{13}$C-NMR (DMSO, 100 MHz): 173.22, 158.14, 155.55, 146.57, 142.90, 134.84, 125.99, 124.67, 120.21, 118.69, 117.72, 106.69.

**Sulfated compound 10 (10s).** $^{1}$H-NMR (DMSO, 400 MHz): 8.14 (m, 1 H), 7.64 (m, 2 H), 7.32 (m, 1 H), 7.21 (m, 1 H), 6.49 (m, 1 H). $^{13}$C-NMR (DMSO, 100 MHz): 175.70, 160.60, 157.36, 152.92, 147.13, 143.82, 123.99, 120.39, 119.78, 117.46, 110.85, 108.07, 106.81, 101.69, 39.57.

$^{1}$H-NMR of compound 8$s$ dissolved in DMSO shows a peak at 12.36 ppm, which is not observed when it is dissolved in D$_2$O (Figures 4.19 and 4.20). This is indicative of the presence of one hydroxyl group that remains after sulfation. When dissolved in D$_2$O, the hydroxyl gets exchanged with deuterium (of D$_2$O) and hence does not appear on the NMR spectrum. This could be the –OH at C-5 as it tends to hydrogen bond with adjacent
ketone group, leading to the formation of a six-membered ring. Therefore, difficulties in sulfating this hydroxyl group causes for end product to contain (n-1) sulfated compound along with persulfated compound, where n = number of hydroxyl groups on the precursor.

4.3.1 Materials and Methods

4.3.2 RP-IP UPLC-MS equipment and experimental conditions

Refer to section 3.3.3.3., chapter 3.

4.3.3 Results and Discussion

Similar to previously discussed sulfated molecules, 8s, 9s and 10s are poorly retained on the column under normal UPLC-MS conditions, due to hydrophilicity of acidic sulfate groups. Figure 4.12 shows the elution of 8s along with the solvent peak, at 0.3 minutes and an MS spectrum devoid of peaks, except for those from the solvent (Figure 4.13). On the other hand, in the presence of 25 mM HXA, the structural isomers are well retained (Figure 4.14), eluting not before 2 minutes (k’ ~ 6).

RP-IP UPLC-MS at cone voltages 20, 40, and 60 V give unique and reproducible fragmentation patterns (Figures 4.15 to 4.17). Table 8 identifies all the peaks observed in the spectra, with their calculated and observed m/z values. The isomers lost their sulfate groups at increased cone voltages to give peaks of the same m/z, but these losses occurred at different intensities. i.e. some losses were more pronounced in one isomer and less in the other (Table 9). The reproducible pattern of these intensities is what forms the essence of this MS fingerprint. Results obtained from duplicate runs performed in the
same RPIP UPLC-MS conditions showed the difference between 8s, 9s and 10s in the intensities of adduct ions formed after sulfonate-HXA losses, relative to the molecular adduct ion (Figure 4.18).

Differences in sulfonate loss intensities amongst the three positional isomers tested may be due to structural factors such as steric hindrances or even physical factors such as shape of the ion droplet formed and its stability in the MS ion source. It is interesting to note that these differences are present and reproducible only when ion-pairing agents are used in their UPLC-MS characterization. As a result, the losses of sulfonate groups are unique for each isomer, hence, [M - SO₃]: [M] ratios can be used to accurately identify unknown isomers. Collection of such MS fingerprint data for a large number of samples can be used to compile an MS library.

Since our compounds have four sulfate groups each, it is hard to make any conclusions regarding which sulfonate(s) group is lost when looking at the spectrum but similar studies on mono and di-sulfated isomers can give us fundamental understanding of regions on the scaffold where a sulfate group is most vulnerable to MS loss. This information can lead us to understanding the number and positions of sulfate groups in HS GAGs and therefore unlock the key to their specific and non-specific binding affinities to proteins in our body. Analysis of sulfated molecules for purity, degradation or identification of isomeric forms using our RP-IP UPLC-MS method can give us knowledge of stable and unstable sulfate groups, helping design better heparin mimics. Further studies involving analysis of sulfated small molecules in plasma are predicted to help us understand their behavior in-vivo.
Figure 4.12. UV chromatogram and mass chromatogram respectively (from top), for molecule 8s in the absence of ion-pairing agents, Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V. No retention observed as 8s elutes with the solvent peak at 0.3 minutes.
Figure 4.13. Corresponding mass spectrum for molecule 8s in the absence of ion-pairing agents, Solvent A, water + 0.1% v/v formic acid; Solvent B, acetonitrile + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 4.14. Chromatograms of 9s, 10s and 8s respectively (from top), in the presence of 25 mM HXA. Retention increased from 0.3 to 2.2 minutes; isomers show minute differences in elution times. Solvent A, water containing 25 mM HXA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM HXA + 0.1% v/v formic acid; MS capillary voltage, 4 kV; desolvation temperature, 350°C and gas flow, 650 L/hr.
Figure 4.15. MS fingerprint of molecule 8s at cone voltages 20 V (top), 40 V (middle) and 60 V (bottom).
Figure 4.16. MS fingerprint of molecule 9s at cone voltages 20 V (top), 40 V (middle) and 60 V (bottom).
Figure 4.17. MS fingerprint of molecule 10s at cone voltages 20 V (top), 40 V (middle) and 60 V (bottom).
Table 8. Mass spectral data obtained by RPIP UPLC-MS of sulfated positional isomers, 8s; 9s; and 10s, in increasing order of cone voltage conditions

<table>
<thead>
<tr>
<th>Cone voltage (V)</th>
<th>Peak</th>
<th>m/z formula</th>
<th>Calculated m/z</th>
<th>Observed m/z</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>8s</td>
<td>9s</td>
</tr>
<tr>
<td>20</td>
<td>M_1</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA]^{1+}</td>
<td>1114.50</td>
<td>1113.07</td>
</tr>
<tr>
<td></td>
<td>M_{1a}</td>
<td>[M_1]^{1+} - 1 HXA + 1 H^+</td>
<td>1013.37</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M_2</td>
<td>[M_1]^{1+} - 1 HXA - SO_3^- + 1 H^+</td>
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<td>932.43</td>
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<td></td>
<td>M_1</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA]^{1+}</td>
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<td>1113.00</td>
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<tr>
<td></td>
<td>M_{1a}</td>
<td>[M_1]^{1+} - 1 HXA + 1 H^+</td>
<td>1013.37</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M_2</td>
<td>[M_1]^{1+} - 1 HXA - SO_3^- + 1 H^+</td>
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<td>932.59</td>
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<tr>
<td></td>
<td>M_{2a}</td>
<td>[M_2]^{1+} - 2 HXA + 2 H^+</td>
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<td>830.95</td>
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<td>M_3</td>
<td>[M_2]^{1+} - 2 HXA - 2 SO_3^- + 2 H^+</td>
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<td>750.68</td>
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<td>[M_3]^{1+} - 3 HXA + 3 H^+</td>
<td>651.11</td>
<td>649.36</td>
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<tr>
<td>40</td>
<td>M_1</td>
<td>[(M – 4 Na + 4 HXA) + 1 HXA]^{1+}</td>
<td>1114.50</td>
<td>1112.88</td>
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<td></td>
<td>M_{1a}</td>
<td>[M_1]^{1+} - 1 HXA + 1 H^+</td>
<td>1013.37</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M_2</td>
<td>[M_1]^{1+} - 1 HXA - SO_3^- + 1 H^+</td>
<td>933.37</td>
<td>931.58</td>
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<tr>
<td></td>
<td>M_{2a}</td>
<td>[M_2]^{1+} - 2 HXA + 2 H^+</td>
<td>832.24</td>
<td>830.63</td>
</tr>
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<td>M_3</td>
<td>[M_2]^{1+} - 2 HXA - 2 SO_3^- + 2 H^+</td>
<td>752.24</td>
<td>750.72</td>
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<td>[M_3]^{1+} - 3 HXA + 3 H^+</td>
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<td>649.40</td>
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<tr>
<td>60</td>
<td>M_2</td>
<td>[M_1]^{1+} - 1 HXA - SO_3^- + 1 H^+</td>
<td>933.37</td>
<td>931.58</td>
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<td>M_{2a}</td>
<td>[M_2]^{1+} - 2 HXA + 2 H^+</td>
<td>832.24</td>
<td>830.63</td>
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<tr>
<td></td>
<td>M_3</td>
<td>[M_2]^{1+} - 2 HXA - 2 SO_3^- + 2 H^+</td>
<td>752.24</td>
<td>750.72</td>
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<td></td>
<td>M_{3a}</td>
<td>[M_3]^{1+} - 3 HXA + 3 H^+</td>
<td>651.11</td>
<td>649.40</td>
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<td></td>
<td>M_4</td>
<td>[M_3]^{1+} - 3 HXA - 3 SO_3^- + 3 H^+</td>
<td>571.11</td>
<td>569.43</td>
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</table>
Table 9. MS signal intensity-based fingerprint data of peaks M1, M2 and M3 of 8s, 9s and 10s, at cone voltage, 40 V.

<table>
<thead>
<tr>
<th>Peak (m/z)</th>
<th>Average Peak Intensity (run 1, run 2) at 40 V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8s</td>
</tr>
<tr>
<td>M1 (1113.0)</td>
<td>4.7 e^6 (4.3 e^6; 5.0e^6)</td>
</tr>
<tr>
<td>M2 (932.5)</td>
<td>1.5 e^6 (1.5 e^6; 1.4 e^6)</td>
</tr>
<tr>
<td>M3 (750.5)</td>
<td>5.7 e^5 (5.2 e^5; 6.2 e^5)</td>
</tr>
</tbody>
</table>
Figure 4.18. Percentages of [M - 1 SO₃⁻], M₂; and [M - 2 SO₃⁻], M₃ ions, relative to molecular adduct ions (M) i.e. 8s, 9s and 10s (A through C). MS fingerprint; cone voltage, 40 V. Percentage losses were calculated as the average from 2 runs, standard deviation indicated by error bars.
Figure 4.19. $^1$H-NMR of 8s in D$_2$O
Figure 4.20. $^1$H-NMR of 8s in DMSO
Figure 4.21. $^{13}$C-NMR of 8s in DMSO
Figure 4.22. $^1$H-NMR of 9s in DMSO
Figure 4.23. $^{13}$C-NMR of 9s in DMSO
Figure 4.24. $^1$H-NMR of 10s in DMSO
Figure 4.25. $^{13}$C-NMR of 10s in DMSO
CHAPTER 5

RPIP UPLC-MS Of Sulfated Penta O-Galloyl D-Glucopyranose (SPGG)

Biochemical screening of a focused library of sulfated, small molecules belonging to the flavonoid, tetrahydroisoquinoline, cinamic acid, and gallic acid series led to the identification of SPGG, an aromatic heparin mimetic, now known to possess eight to eleven sulfate groups. SPGG inhibited factor XIa with an IC$_{50}$ of 626 nM, which was at least 200-fold better than that for factors IIa, Xa, IXa and XIIa. SPGG prolonged human plasma clotting time selectively in the activated partial thromboplastin assay and prevented whole blood clotting at concentrations as low as 26 μM. Michaelis–Menten kinetic studies performed by the Desai lab showed that SPGG induced a significant reduction in the $V_{\text{MAX}}$ without much affecting the apparent $K_{M}$ suggesting an allosteric mechanism of factor XIa inhibition. The presence of unfractionated heparin reduced the IC$_{50}$ of SPGG, which matched the loss in potency predicted on the basis of ideal competition between the two allosteric ligands. No chemically synthesized molecule has been discovered so far that exhibits such high potency for FXIa inhibition coupled with an allosteric mechanism.$^{18}$

Aside from the parent PGG core and the presence of sulfate groups, the structure of SPGG was relatively unknown, prior to UPLC-MS analysis. The globular core structure of PGG makes it highly flexible and presumably difficult to sulfate all the 15 hydroxyl groups attached. As a result, the end product of this partial sulfation is a mixture of variedly sulfated SPGG compounds (see schematic in Figure 5.1). In this chapter, we
discuss the characterization of this complex mixture using the RPIP UPLC-MS method that we successfully used for the compounds discussed in the previous chapters. Also, we use up-front CID, for the first time on an unknown mixture, to deduce important structural information pertaining to SPGG. This practical application provides as a validation of sorts for the versatility of our RPIP UPLC-MS method in characterization of simple small molecules to larger complex mixtures.

![Chemical Structure](image)

**Figure 5.1.** Schematic of sulfation of PGG to SPGG; R = -OH, -\( \text{OSO}_3 \)

### 5.2 Experimental methods

#### 5.2.1. RP-IP UPLC-MS equipment and experimental conditions

SPGG synthesized in our lab was used and a 500 μM solution was made by dissolving in water. 5 μL of this sample was injected onto the column. A 26 minute run was performed at a shallow gradient starting with 20% solvent B, rising linearly to 80% in 20 minutes (3% per min). This was to ensure maximum column separation of closely
related SPGG components. All other conditions were maintained the same as mentioned in earlier chapters (Refer to chapter 3, section 3.3.3.3).

5.3 Results and Discussion

Structure Determination of Sulfated Pentagalloyl Glucopyranoside (SPGG).

The capillary electrophoretic profile of SPGG in reverse polarity mode displayed a complex, ill-resolved pattern indicating the presence of partially sulfated components (not shown). To identify the proportion and structure of these components, we resorted to reversed-phase ion-pairing UPLC-MS. In this technique, the ion-pairing agent, n-hexylamine, is introduced in the mobile phase so as to replace sodium cations present on each sulfate group and impart considerable hydrophobicity to the molecule. Resolution arises from the different hydrophobicities of the constituents that contain varying number of n-hexylamine groups.

Under normal UPLC-MS conditions, SPGG, owing to its high hydrophilicity, elutes as a broad peak in 0.29 minutes (see Figure 5.2). Ion-pairing with 25 mM HXA under a shallow gradient resulted in a UPLC profile of SPGG that showed the presence of five major nearly baseline resolved peaks, labeled \( p1 \) through \( p6 \) in Figure 5.3, each of which is further composed of multiple peaks.

The ESI-MS profile of each peak, observed between 1000 and 2048 \( m/z \) range, was found to contain a doubly charged molecular adduct ion with a general formula of \([\text{PGG}+x\times\text{SO}_3\text{-HXA}–x\times\text{H}]+2\times\text{HXA}]^{2+}\), where \( x \) is the number of sulfonate (SO\(_3\))-hexylammonium (HXA) groups present in the molecule (see Figures 5.6 through 5.11).
For example, peaks $p3$, $p4$ and $p5$ displayed molecular adduct ions at 1388.43, 1478.99 and 1569.60 m/z, respectively, corresponding to doubly charged SPGG species containing 9, 10 and 11 sulfate groups with 11, 12 and 13 hexylamines, respectively, as ion-pairs. A similar behavior was observed for peaks $p1$, $p2$ and $p6$, which corresponded to SPGG species with 7, 8 and 12 sulfate groups, respectively. In addition to the molecular adduct ions, each of these peaks also displayed several other ions that arose due to the loss of one or more hexylamine-paired sulfonate groups further confirming the identity of parent sulfated species (Figures 5.6 to 5.10).

Each of these peaks ($p1$ through $p6$), is a complex peak comprising of minor peaks. To further identify the origin of multiple components observed in peaks $p1$ through $p6$, we utilized selective ion recording (SIR)–MS. In this technique, the spectrometer is tuned to monitor a specific ion, e.g., 1478.99 m/z corresponding to $[\text{M+10 SO}_3+12 \text{ hexylamines}]^{2+}$ ion, resulting in the identification of all peaks that contain this ion. Figure 5.4 shows three SIR profiles of SPGG. Monitoring at 1388.43 m/z gave a SIR profile that essentially mimicked peak $p3$ of the UV chromatogram suggesting that each component present in $p3$ contained nine sulfate groups. Likewise, monitoring at 1478.99 or 1569.90 m/z resulted in a profile equivalent to the chromatogram of $p4$ or $p5$, respectively. This was also found to be the case for peaks $p1$, $p2$ and $p6$ (see Figures 5.4 and 5.5). Figure 5.11 shows the division of peak $p4$ into 4 major arms or minor peaks; a, b, c, d.

Mass spectra of the minor peaks within a given complex peak were observed at 20 and 60 V. Figure 5.12 shows mass spectra of minor peaks of peak $p4$ at cone voltage
equal to 20 V. At 20 V, spectrum of each minor peak shows a single ion signal of the same m/z, i.e., 1479, corresponding to the molecular adduct ion. At 60 V, each spectrum showed additional ion peaks, arising from controlled fragmentation of molecular adduct ion (see Figure 5.13). The mass difference between any peak and its consecutive peak, on all mass spectra, was approximately equal to 91. Using the formula (see below, detailed in Chapter 4) to calculate peak charge, we found that, indeed, these ions carry a charge of +2, which matched with the mass differences.

According to Figure 5.13 (mass spectrum at CV 60),

\[ M_1 - M_2 = [(PGG + (x) SO_3-HXA) + 2 HXA]^{2+} - [(PGG + (x-1) SO_3-HXA + x H) + 2 HXA]^{2+} \approx 91 \]

Where \( x = \) number of sulfonate-HXA groups

And charge,

\[ n = \frac{\text{mass of sulfonate-IPA} - 1H}{M_1 - M_2} \]

\[ = \frac{181.13}{91} \approx 2 \]

Therefore, \( n = 2 \). Using this method, we were able to deduce the identity and charge of the peaks \( p1 \) through \( p6 \) by looking at the pattern of losses on each spectra.
Additionally, we observed that just like positional isomers 8s, 9s and 10s, the minor peaks within each peak, showed unique sulfonate loss intensity patterns, confirming that they represent positional isomers, each with a reproducible mass spectral fingerprint.

In conclusion, UPLC-MS analysis of three independently prepared batches suggested that SPGG is a mixture of septa- ($p_1$), octa- ($p_2$), nona- ($p_3$), deca- ($p_4$), undeca- ($p_5$) and dodeca- ($p_6$) sulfated species, which further contain species with an identical number of sulfate groups (see Figure 5.14). It is likely that major peaks are partially resolved into complex peaks because of sulfate positional isomerism. This enhances the structural diversity of SPGG. Peaks $p_1$ through $p_6$ are present in proportions of 0.06:0.17:0.21:0.45:0.11:0.03, respectively (Figures 5.4 and 5.5). Using these proportions and the associated molecular weights, the weight-average molecular weight of SPGG was calculated to be 2668 (hexylammonium ion form) or 2178 (Na$^+$ form).
**Figure 5.2.** UV chromatogram of UPLC-MS of SPGG in the absence of ion-pairing agents. Two peaks are seen; solvent peak, at 0.21 minutes; and SPGG at 0.29 minutes. Solvent A, water containing 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 0.1% v/v formic acid; cone voltage, 20 V, t = 26 minutes.
Figure 5.3. RPIP UPLC-UV spectrum of SPGG in the presence of 25 mM HXA; Solvent A, water containing 25 mM PTA + 0.1% v/v formic acid; Solvent B, acetonitrile; water (3:1) containing 25 mM PTA + 0.1% v/v formic acid; cone voltage, 20 V, t = 26 minutes.
Figure 5.4. SIR of peaks $p_3$, $p_4$ and $p_5$ of SPGG. (A) shows UPLC resolution of SPGG into six peaks ($p_3$ to $p_5$), which arise from variable sulfation of PGG. (B) – (D) show SIR monitoring of SPGG at 1388, 1479 and 1569 m/z to identify the peaks corresponding to 9, 10 and 11 sulfated PGG species. SIR = selective ion recording; See text for detailed interpretation.
Figure 5.5. SIR of peaks $p_1$, $p_2$ and $p_6$ of SPGG. (A) shows UPLC resolution of SPGG into six peaks ($p_1$ to $p_6$), which arise from variable sulfation of PGG. (B) – (D) show SIR monitoring of molecule 6 at 1207, 1296 and 1660 m/z to identify the peaks corresponding to 7, 8 and 12 sulfated PGG species. SIR = selective ion recording; See text for detailed interpretation.
Figure 5.6. ESI-MS spectrum of peak \( p6 \) at 13.096 min in the range 200 – 2048 amu using a scan time of 0.25 s. PGG is with a mass of 940.68, HXA = \( n \)-hexylammonium ion having a mass of 102.13, and SO\(_3\) is a sulfonate group with a mass of 79.96. \( p6: \quad [(\text{PGG} + 12 (\text{SO}_3\text{-HXA}) - 12 \text{ H}) + 2 \text{ HXA}]^{2+} \).
Figure 5.7. ESI-MS spectrum of peak p5 at 11.934 min in the range 200 – 2048 amu using a scan time of 0.25 s. PGG is with a mass of 940.68, HXA = \( n \)-hexylammonium ion having a mass of 102.13, and SO3 is a sulfonate group with a mass of 79.96. (p5: \[((PGG + 11 (SO3-HXA)–11 H) + 2 HXA)\] \(^{2+}\)).
Figure 5.8. ESI-MS spectrum of peak $p_4$ at 10.773 min in the range 200 – 2048 amu using a scan time of 0.25 s. PGG is with a mass of 940.68, HXA = $n$-hexylammonium ion having a mass of 102.13, and SO$_3$ is a sulfonate group with a mass of 79.96. ($p_4$: $[(\text{PGG} + 10 (\text{SO}_3\text{-HXA}) - 10H)+2 \text{ HXA}]^{2+}$).
Figure 5.9. ESI-MS spectrum of peak $p_3$ at 9.934 min in the range 200 – 2048 amu using a scan time of 0.25 s. PGG is with a mass of 940.68, HXA = $n$-hexylammonium ion having a mass of 102.13, and SO$_3$ is a sulfonate group with a mass of 79.96. ($p_3$: [(PGG + 9 (SO$_3$ - HXA) – 9 H) + 2 HXA]$^{2+}$).
Figure 5.10. ESI-MS spectrum of peak p2 at 9.127 min in the range 200 – 2048 amu using a scan time of 0.25 s. PGG is with a mass of 940.68, HXA = n-hexylammonium ion having a mass of 102.13, and SO$_3$ is a sulfonate group with a mass of 79.96. (p2: [(PGG +8 (SO$_3$-HXA) – 8 H) + 2 HXA] $^{2+}$).
Figure 5.11. RPIP UPLC-MS chromatogram of SPGG in the presence of 25 mM HXA (above); single ion count chromatogram for peak p4 (below); [PGG + 10 SO$_3$ + 12 HXA]$^{2+}$, m/z = 1479. Solvent A, water containing 25 mM PTA + 0.1% v/v formic acid; Solvent B, acetonitrile: water (3:1) containing 25 mM PTA + 0.1% v/v formic acid; cone voltage, 20 V.
Figure 5.12. Mass spectra corresponding to minor peaks a, b, c, d of $p4$, at cone voltage = 20 V. Base peaks in all spectra are of the same $m/z \approx 1479$, indicative of positional isomeric peaks.
Figure 5.13. Mass spectral fingerprints corresponding to minor peaks a, b, c, d of p4, at cone voltage = 60 V.
Figure 5.14. Comparison of the UPLC-MS profiles of three batches of SPGG prepared independently.
Conclusions:

- Retention and identification of small molecules 1 to 5 as intact ions was achieved through reversed-phase ion-pairing UPLC-MS.

- Comparative analysis showed increments in $k'$ values of 1 to 5 with different agents as being dependent on number of sulfates.

- We hypothesize that the number of sulfates in a unknown molecule can be identified by slope values obtained by plotting $k'$ vs. carbon atom number of ion-pairing agent.

- Ion-pairing was found to be more structure-dependent than dependent on carbon atom number/alkyl number of ion-pairing agent.

- Collision-induced dissociation or cone voltage-based fragmentation was effectively used in:
  - identifying minimum number of sulfates on the molecule
  - the development of unique fragmentation patterns called “fingerprints”
  - the separation of positional isomers and obtained unique MS fingerprints for each
  - identifying unknown compounds as multiply-charged ions
• Successfully characterized sulfated pentagalloyl glucopyranoside mixture and identified the presence of nona-, deca- and undeca- sulfated SPGG species, quantified them relative to the other species.

• Overall, no derivatization methods such as permethylation were required to obtain mass information on sulfated small molecules.

**Future directions:**

With this project, we aim to characterize β-O-4 lignin polymer, a potent selective thrombin inhibitor discovered by our lab, as well as, other polydisperse sulfated mixtures showing heparin mimetic actions, using ion-pairing UPLC-MS. Another prospective area of research would be the study of positional isomers and identification of sulfate group positions in GAG oligosaccharides, using up-front CID in MS. Furthermore, to expand our panel of ion-pairing agents, polyminoalkanes, such as spermine and spermidine, secondary amines, etc. would, in the future, be tested against our library of molecules. In the future, we aim to study the interaction of GAG mimetics with ion-pairing agents as a representative model of their binding site interactions with ligands in the body.


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