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Quantitative determination of disaccharide content in digested unfragmented heparin and low molecular weight heparin by direct-infusion electrospray mass spectrometry

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Abstract

Heparins and low molecular weight heparins (LMWHs) are heterogeneous glycosaminoglycans derived from natural sources that are prescribed as anticoagulants. In this work, a direct-infusion electrospray ionization mass spectrometry (ESI-MS) method was applied to the quantitative analysis of known disaccharides in various native heparins and LMWHs after digestion with heparinase enzymes. Disaccharide ΔUA2S → GlcNS6S was found to compose the majority of all samples analyzed (81–88%). The values were significantly higher than those reported by previously published methods. The disaccharide isomer pair ΔUA → GlcNS6S/ΔUA2S → GlcNS was also detected in all samples at lower levels (11–19%). While digestion with heparinases I and II revealed a limited number of disaccharides, the addition of heparinase III to digests led to the detection of disaccharide ΔUA2S → GlcNAc6S in native porcine heparin. This result indicated the importance of utilizing all three heparinases to gain maximum information when analyzing heparin and LMWH digests. This method displayed good between-day (4–6%) and between-digest (1–2%) reproducibility in separate experiments. To determine if the digestion matrix was suppressing the signal of low-abundance disaccharides, several disaccharides were exogenously added at low levels (1–10 pmol/mg) to a quenched digest reaction. Analysis revealed that low level disaccharides were detectable in this matrix above the limits of detection (0.1–0.2 pmol/mg) and quantitation (0.2–0.7 pmol/mg). While this method was unable to distinguish between disaccharide isomers, it utilized simple mass spectrometry instrumentation to provide useful quantitative data for characterizing preparations of native heparin and LMWH, which could be used to compare various marketed preparations of these popular anticoagulants.

Keywords: Heparin; LMWH; Electrospray ionization mass Spectrometry; Disaccharide; Heparinase; Relative quantitation

1. Introduction

Heparin and low molecular weight heparin (LMWH) are heterogeneous glycosaminoglycans (GAG) prescribed as anticoagulants. Heparin contains a pentasaccharide sequence [GlcNAc/NS(6S) → GlcUA → GlcNS(3S, 6S) → IdoA(2S) → GlcNS(6S)] which binds to antithrombin III (AT), inhibiting the coagulation cascade. Between 20 and 50% of GAG chains present in pharmaceutical grade heparin contain the AT binding site, with structural variability existing both within and outside of the pentasaccharide sequence. LMWHs are a newer class of anticoagulants with increased bioavailability and are prepared by the controlled depolymerization of native, unfragmented pharmaceutical grade heparin. Various depolymerization reactions can be utilized to prepare LMWHs, including oxidation, deaminative degradation, and chemical or enzymatic β-elimination [1]. Each of these processes results in LMWHs with unique chemical compositions that possess differing clinical safety and efficacy profiles [2,3]. In addition, differing animal and tissue sources of the native heparin precursor can lead to variation among LMWH preparations [4]. Currently, porcine intestinal mucosa is a common source for pharmaceutical heparins and LMWHs.

The chemical characteristics of native heparins and LMWHs are complex due to the polydispersity of the linear glycosaminoglycan chains, which contain a basic disaccharide repeat unit composed of sulfated glucosamine and hexuronic acid sugar residues (Fig. 1). Heparin is more highly sulfated than the related GAG, heparan sulfate. The diverse biological roles that these related GAGs play have been attributed to their distinctively

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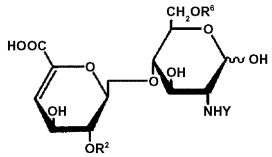

	Disaccharide	R^2	R^6	Y	*m/z*	*z*
IA	ΔUA2S→GlcNAc6S	SO_3	SO_3	Ac	179	-3
IIA	ΔUA→GlcNAc6S	H	SO_3	Ac	229	-2
IIIA	ΔUA2S→GlcNAc	SO_3	H	Ac	229	-2
IVA	ΔUA→GlcNAc	H	H	Ac	378	-1
IS	ΔUA2S→GlcNS6S	SO_3	SO_3	SO_3	288	-2
IIS	ΔUA→GlcNS6S	H	SO_3	SO_3	248	-2
IIIS	ΔUA2S→GlcNS	SO_3	H	SO_3	248	-2
IVS	ΔUA→GlcNS	H	H	SO_3	208	-2
IP	ΔUA2S→GlcNCOEt6S	SO_3	SO_3	COEt	276	-2

SO_3- sulfate; Ac- acetyl; COEt- carboxyethyl

Fig. 1. The backbone structure of a heparin disaccharide is represented. The various side groups are listed for standard heparin disaccharides and the internal standard disaccharide. The *m/z* values chosen for identification and relative quantitation in this work are shown.

sulfated saccharide sequences [5,6]. However, analysis and characterization of such sequences is complicated and has been limited due in part to the labile nature of the sulfates and the high charges that accompany these molecules. Characterization of LMWH includes determination of the polydispersity of particular preparations [7] as well as the identification of unique end-groups that may form during the various depolymerization processes [8]. The heterogeneous polysaccharide chains of native heparins and LMWHs can be digested into disaccharides by several heparinase enzymes of varying specificities derived from the bacterium, *Flavobacterium heparinum* [9–11]. The cleavage specificities of these heparinase enzymes (I, II, and III) have been characterized and are particular to linkages found in either heparin or heparan sulfate, as detailed in Table 1. One approach to characterizing LMWHs is to quantitatively determine the sulfated disaccharide profile contained in these simplified heparinase digests. This type of data provides information on levels of overall disaccharide sulfation, as well as possible contamination of pharmaceutical native heparin and LMWH preparations with other, less sulfated GAGs. Much of the work previously performed to determine relative heparin disaccharide content has utilized various forms of separation, including capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC), often coupled to UV absorbance detection [12–16]. More recently, several groups have utilized tandem mass spectrometry (MS^n) without any prior sample separation to analyze and quantitate relative proportions of various disaccharides in heparin disaccharide standard mock mixes or heparin digests [17,18]. While qualitative analysis of derivatized heparin disaccharides has been achieved with HPLC coupled to mass spectral detection [19], this method has not alleviated the necessity of MS^n for distinguishing between isomers, since complete separation of the various disaccharides was not achieved by utilization of graphitized carbon chromatography. In addition, the yield of individual derivatized disaccharides from this method was highly variable and internal standards were not incorporated, precluding quantitation. The work presented here utilized available heparin disaccharide standards and adapted a direct-infusion electrospray ionization mass spectrometry (ESI-MS) method [17] to identify and quantify the relative proportions of sulfated disaccharides in digests of native heparin and LMWH samples from various sources. In addition, the possibility of a matrix effect suppressing the mass spectral signal of low-abundance heparin disaccharides was investigated. The heparin disaccharides analyzed in this work differ in mass, with the exception of two pairs of isomers (IIS/IIIS and IIA/IIIA),

Table 1
Specificity of various heparinase enzymes

Heparinase	Cleaved linkage	Source of cleaved linkage
I	α-D-Deoxy 2-aminoglucopyranose$_{NS,6X}$–α-L-idopyranosyluronic acid$_{2S}$	Heparin
II	α-D-Deoxy 2-aminoglucopyranose$_{NY,6X}$–α-L-idopyranosyluronic acid$_{2X}$	Heparin/heparan sulfate
	α-D-Deoxy 2-aminoglucopyranose$_{NY,6X}$–β-D-glucopyranosyluronic acid$_{2X}$	Heparin/heparan sulfate
III	α-D-Deoxy 2-aminoglucopyranose$_{NAc}$–α-L-idopyranosyluronic acid	Heparan sulfate
	α-D-Deoxy 2-aminoglucopyranose$_{NY,6X}$–β-D-glucopyranosyluronic acid	Heparan sulfate

2S, 2-*O* sulfation; 6S, 6-*O* sulfation; NS, *N*-sulfation of glucosamine; X, *O*-sulfation or no substitution; Y, *N*-sulfation or *N*-acetylation.

making this ESI-MS method feasible. This method required no purification or separation of heparin digest reactions prior to analysis and may be useful for rapid differentiation among various marketed native, unfragmented heparins and LMWHs.

2. Experimental

The direct-infusion ESI-MS method applied in this report was chosen based on its ability to quantify the relative proportion of various known disaccharides in native heparin and LMWH digests with minimal sample processing prior to analysis [17]. Commercially available heparins and LMWH were digested with multiple heparinases in the presence of an internal standard, the heparin disaccharide analog, IP. The final solvent composition of the digests was adjusted to methanol–water (50:50, volume fraction) with ammonium hydroxide (0.01 mol/L) for compatibility with direct-infusion ESI-MS analysis in negative mode. Mass spectral peaks corresponding to disaccharide standards were identified and the ratio of peak heights (disaccharide:internal standard) were determined and fit onto linear calibration curves established from standard disaccharide mixtures.

2.1. Materials

Heparin disaccharide standards IS, IIS, IA, IIA, and IVA were purchased from V-Labs, Inc. (Covington, LA). IVS was obtained from Grampian Enzymes (Orkney, UK) and IP (99% purity) from Calbiochem (La Jolla, CA). Heparin from porcine intestinal mucosa (H-6279), LMWH from porcine intestinal mucosa (H-3400), heparin from bovine intestinal mucosa (H-0777), and heparinases I, II, and III were all purchased from Sigma–Aldrich, Co. (St. Louis, MO). Additional heparinases I and II stocks were kindly provided by IBEX Technologies Inc. (Montreal, Quebec, Canada). All other reagents and solvents were purchased from commercial sources. Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the NIST, nor does it imply that the materials or equipment identified are the best available for the purpose.

2.2. Instrumentation

Mass spectra were acquired on a Micromass ZMD single-quadrupole mass spectrometer equipped with an electrospray ionization probe in negative ion mode utilizing MassLynx v3.5 software. Standard calibrant solutions and digest reactions were introduced into the mass spectrometer by direct infusion at 10 μL/min. The following instrumental parameters were used in acquiring all mass spectra: capillary voltage, 3.00 kV; cone voltage, 5 V; extractor voltage, 5 V; radio frequency (RF) lens, 0.10 V; source block temperature, 80 °C; desolvation temperature, 120 °C; ion energy, 0.5; low mass (LM) resolution, 15.0; high mass (HM) resolution, 15.0; multiplier, 650 V; scan cycle time, 0.800 s; scan duration, 0.75 s; interscan delay, 0.05 s; retention window, 0.000 to 1.000 min; *m*/*z* range, 150–500.

2.3. Preparation and storage of materials

All heparin disaccharide stocks were gravimetrically prepared by diluting powder stocks with methanol–water (50:50, volume fraction) with ammonium hydroxide (0.01 mol/L) and were stored at −20 °C. All disaccharide concentrations are noted as pmol of disaccharide per mg of total solution (pmol/mg). Heparin and LMWH stocks were prepared by diluting powder stocks with MilliQ water to 2.7 mg/mL and were stored at −20 °C. Heparinase I, II, and III (Sigma) stocks were prepared by diluting powder stocks to 0.001 IU (1 IU produces 1 μmol unsaturated uronic acid/min at 37 °C)/μL with ammonium acetate (pH 7.5; 0.1 mol/L). Additional heparinases I and II stocks (IBEX) were also diluted to 0.001 IU/μL in the same solvent as above from concentrated, buffered stock solutions. Heparinase I–heparinase II stocks were combined (1:1, volume fraction) or heparinase I–II–III stocks were combined (1:1:1, volume fraction) to produce heparinase cocktails, which were aliquoted into reaction tubes appropriate for single-digest reactions and stored at −80° C.

2.4. Calibration

Heparin disaccharide calibrants were prepared by serial dilution of a 6-disaccharide stock mixture containing 100 pmol/mg each of disaccharides IS, IIS, IVS, IA, IIA, and IVA to final concentrations of 1, 5, 25, 50, 75, and 100 pmol/mg in methanol–water (50:50, volume fraction) with ammonium hydroxide (0.01 mol/L). All dilutions were carried out volumetrically. Each calibrant solution contained a constant internal standard (IP) concentration of 5 pmol/mg. Disaccharides IIIS and IIIA were omitted from the standard calibrant mixture because they are isomers of IIS and IIA, respectively, and cannot be distinguished by MS alone. Calibrants were stored at −20 °C.

Mass spectra for each calibrant mixture were acquired in triplicate. Calibration curves were created by plotting the concentration of each heparin disaccharide standard versus the mean ratio of disaccharide peak height to the internal standard peak height. These curves were fit to a least squares linear regression for each disaccharide standard.

2.5. Enzyme digests

Digest reactions were performed on 10 μg of either native porcine heparin, native bovine heparin, or porcine LMWH in a total reaction volume of 75 μL containing ammonium acetate reaction buffer (pH 7.5; 0.02 mol/L) and calcium acetate (0.002 mol/L), 0.01 IU each of heparinases I, II, and III, as noted, and 27 pmol of the internal standard, IP. Digest reactions were incubated at 37 °C with agitation for 24 h. To quench the digest reactions and modify the solvent contents to be compatible with direct-infusion ESI-MS, the following components were added to each reaction: 200 μL methanol, 20 μL aqueous ammonium hydroxide (0.2 mol/L), and 105 μL MilliQ water. The final IP internal standard concentration in all heparinase digest reactions after quenching was 5 pmol/mg. Digest reactions were stored at −20 °C prior to analysis. To investigate between-day and between-digest reproducibility, one set of digests was analyzed

on two separate days (digested with heparinases I and II) or two sets of digests were prepared in parallel and analyzed on the same day (digested with heparinases I, II, and III), respectively. Mass spectra from all digests were acquired in triplicate.

2.6. Determination of limits of detection and quantitation

To estimate the limits of detection (LOD) and limits of quantitation (LOQ) for this method, signal-to-noise (S/N) values were first determined at the lowest level of disaccharide standards measured (1 pmol/mg). Estimates for LOD (S/N = 3) and LOQ (S/N = 10) were linearly extrapolated from these values.

2.7. Determination of digest reaction matrix effects on detection of low-abundance disaccharides

To demonstrate that lower levels (1–10 pmol/mg) of the disaccharides IVS, IA, IIA, and IVA, which were not initially observed in native heparin and LMWH digest samples, would be detectable in the matrix of a LMWH digest, a 100 pmol/mg master mix of these disaccharides was prepared in a quenched, LMWH digest matrix. This master mix was serially diluted to final concentrations of 1, 2.5, 5, and 10 pmol/mg in the same digest reaction matrix. These samples were then analyzed by ESI-MS under the conditions stated previously.

3. Results and discussion

The use of a direct-infusion ESI-MS method allowed for the acquisition of reproducible mass spectra from standard disaccharide calibrants and various heparin and LMWH digests, which, in turn, were utilized to identify and quantify the relative proportion of disaccharides present in unfragmented heparin and LMWH samples as one means of characterization.

The mass spectra of the standard calibrants contained at least two characteristic ions for each standard heparin disaccharide, representing the −1, −2, or −3 charge-state ions. Consistent with previously reported observations [17], the varying charge-states appeared to depend on the number of sulfates present in the individual disaccharides, where the presence of increasing numbers of sulfate groups was associated with higher charge-state ions. For most disaccharides, the −2 charge-state ions were utilized, with the exceptions of −3 for IA and −1 for IVA. It is worth noting that the mass-to-charge values chosen in this work (Figs. 1 and 2) for calculating disaccharide proportions were not all identical to those utilized in previous works in which heparin disaccharides were analyzed by MS-based methods [17,18]. Prominent mass peaks not utilized in the final disaccharide calculations include *m/z* 165, 191, and 198 (Figs. 2–4), which represent the −3 charge-state ions of IIS/IIIS, IS, and Na-IS, respectively. In addition, several prominent mass peaks observed in the digestion mixtures (*m/z* 212, 223, and 305) were observed as background peaks in negative control reactions containing only heparinases and buffer (data not shown). Although every attempt was made to reproduce the experimental sample and MS conditions previously reported [17], variation in response between different instruments may be

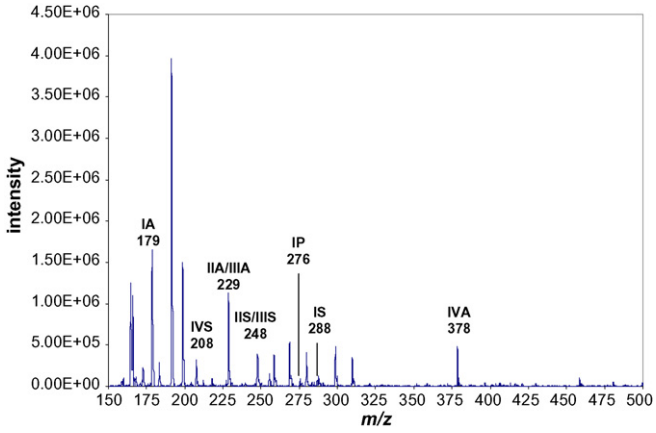

Fig. 2. An example full-scan (*m/z* 150–500) mass spectrum of the disaccharide standard calibrant mix (25 pmol/mg shown here). The peaks utilized for calculating standard calibration curves are labeled with the *m/z* value and the disaccharide(s) that they represent.

responsible for observed differences in the quality of the various charge-state peaks. The *m/z* values utilized in the quantitation calculations were determined empirically as the ions that gave the best-fit linear calibration curves for each standard disaccharide in the 1–100 pmol/mg range (Table 1). Other peaks observed in the acquired *m/z* range (150–500) are consistent with additional charge-state ions. Although sodium adducts could not be eliminated from the samples, they were reduced by the addition of ammonium hydroxide [17]. The intra- and interassay reproducibility of this method displayed minimal effects on intensity variation from the sodium-adducted and non-adducted disaccharides intensities. No adduct ion peaks were chosen to establish standard curves. The calibration curves were fit for a linear response in the narrow interval encompassing 1–100 pmol/mg for each standard disaccharide, with R^2 values equal to or greater than 0.95 and non-zero intercepts (Table 2). The slopes of the linear calibration curves for all A-series disaccharides, each of which contain one acetyl side group, were greater than those of the S-series disaccharides. Within the A-series, the presence of increasing numbers of sulfate side groups was also associated with increased slope. The S-series disaccharides that contained either one or two sulfate side groups (IVS and IIS/IIIS, respectively) had similar slopes (≈0.1). The trisulfated disaccharide, IS, displayed the lowest slope of all the calibrants. However, it should be noted that the *m/z* values chosen in this work for calcu-

Table 2
The results of regression analysis on linear calibration curves

Disaccharide	Regression equation, $y = a + bc$[a]	Correlation coefficient	Linear range (pmol/mg)
IS	$y = 0.433 + 0.021c$	0.95	1–100
IIS/IIIS	$y = 0.554 + 0.096c$	0.95	1–100
IVS	$y = -0.135 + 0.100c$	0.95	1–100
IA	$y = -1.60 + 0.532c$	0.95	1–100
IIA/IIIA	$y = -0.554 + 0.326c$	0.95	1–100
IVA	$y = -0.701 + 0.181c$	0.96	1–100

[a] y denotes (disaccharide mass peak intensity/internal standard mass peak intensity); c denotes the concentration (pmol/mg) of the disaccharide analytes.

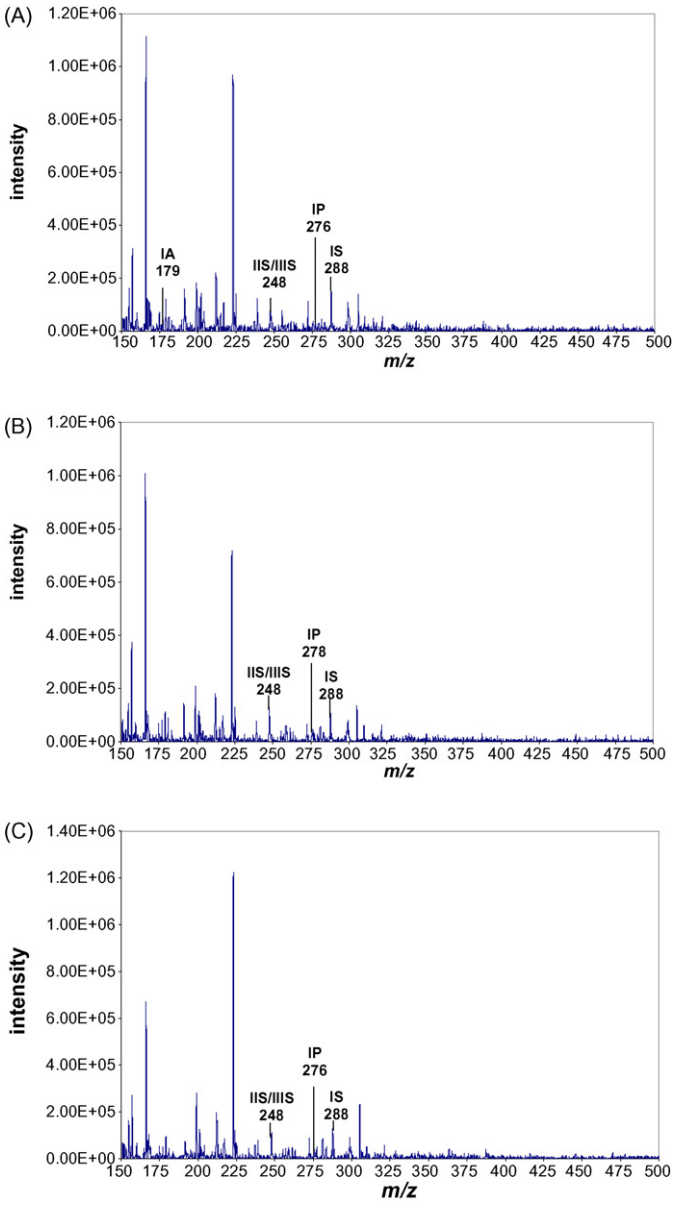

Fig. 3. Representative full-scan (*m/z* 150–500) mass spectra from samples digested with heparinases I, II, and III for (A) porcine intestinal mucosa heparin, (B) bovine intestinal mucosa heparin, and (C) porcine intestinal mucosa LMWH. Peaks representative of standard disaccharides that were utilized in relative proportion calculations, including the internal standard IP, are labeled.

lating calibration curves did not necessarily represent the most intense peaks observed from each disaccharide and, therefore, other charge-states of these disaccharides may display quite dissimilar slopes. All quantitative disaccharide results presented in this work (Table 3) were calculated utilizing the equations derived from calibrant mass spectra obtained on the same day as the digest samples.

3.1. Relative disaccharide content determination of various heparin preparations

The full-scan (*m/z* 150–500) mass spectra from native porcine heparin digested with heparinases I and II displayed mass spectral peaks consistent with the disaccharides IS, IIS/IIIS, and IP internal standard. When digested with all three heparinases, the unfragmented porcine heparin displayed a mass spectral peak consistent with the presence of disaccharide IA in addition to the previously identified disaccharides (Fig. 3A). The mean values from each digest reaction, as well as the combined mean values from duplicate digests, are displayed in Table 2. The proportion of disaccharides in native heparin digested with heparinases I and II ranged from 84 to 88% for IS and 12–16% for IIS. When digested with heparinases I, II, and III, the same

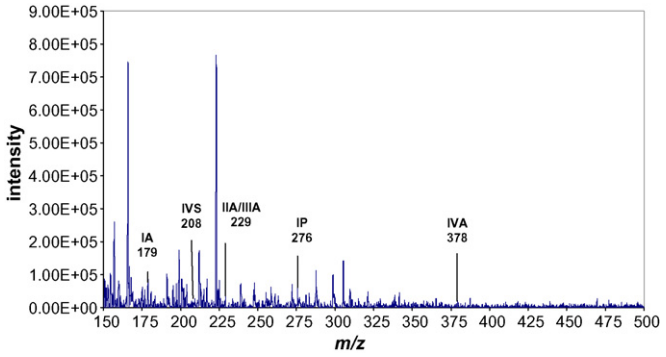

Fig. 4. Representative full-scan (*m/z* 150–500) mass spectrum of a sample containing disaccharides IA, IIA, IVA, and IVS added exogenously to a quenched porcine LMWH digest matrix. Exogenous disaccharides were spiked in at a final concentration of 1 pmol/mg in sample shown. The peaks of spiked-in disaccharide standards are labeled with the *m/z* value and the disaccharide(s) that they represent.

Table 3
Molar percent of standard disaccharides in digested heparin and LMWH samples determined by direct-infusion ESI-MS

Sample	IS	IIS/IIIS	IVS	IA	IIA/IIIA	IVA
Sigma porcine heparin						
Day 1[a,b]	88 ± 0.3	12 ± 0.3	ND	ND	ND	ND
Day 2[a,b]	84 ± 1.3	16 ± 1.3	ND	ND	ND	ND
Combined[c]	86 ± 0.8	14 ± 0.8	ND	ND	ND	ND
Sigma porcine LMWH						
Day 1[a,b]	87 ± 0.5	13 ± 0.5	ND	ND	ND	ND
Day 2[a,b]	81 ± 1.4	19 ± 1.4	ND	ND	ND	ND
Combined[c]	84 ± 0.9	16 ± 0.9	ND	ND	ND	ND
Sigma porcine heparin						
Digest 1[a,d]	83 ± 6.1	12 ± 5.9	ND	5 ± 0.3	ND	ND
Digest 2[a,d]	85 ± 3.8	11 ± 4.2	ND	4 ± 0.9	ND	ND
Combined[c]	84 ± 4.1	12 ± 4.2	ND	5 ± 0.5	ND	ND
Sigma porcine LMWH						
Digest 1[a,d]	88 ± 3.2	12 ± 3.2	ND	ND	ND	ND
Digest 2[a,d]	88 ± 4.6	12 ± 4.6	ND	ND	ND	ND
Combined[c]	88 ± 3.2	12 ± 3.2	ND	ND	ND	ND
Sigma bovine heparin						
Digest 1[a,d]	86 ± 7.3	14 ± 7.3	ND	ND	ND	ND
Digest 2[a,d]	85 ± 0.7	15 ± 0.7	ND	ND	ND	ND
Combined[c]	86 ± 4.2	15 ± 4.2	ND	ND	ND	ND

ND: not detected.
[a] Mean ± standard deviation ($n = 3$).
[b] Digested with heparinases I and II.
[c] Combined mean ± standard error of the mean.
[d] Digested with heparinases I, II, and III.

preparation of heparin was determined to contain IS (83–85%), IIS/IIIS (11–12%), and IA (4–5%).

While previous works [12–17] utilized a mix of all three heparinases in their digests prior to analysis, the work presented here compared the use of heparinases I and II versus I, II, and III for the analysis of heparin and LMWH from porcine intestinal mucosa to determine if the addition of heparinase III had an observable effect on the results. As stated above, digestion of native porcine heparin with heparinases I, II, and III revealed peaks representative of disaccharide IA, which were not observed with heparinases I and II alone. Heparinases I and II have the specificity to cleave heparin into disaccharide units, while heparinase III is expected to digest only heparan sulfate [9–11]. This result may indicate the presence of heparan sulfate contamination in the original porcine heparin preparation, which can commonly occur through the standard purification processes for heparin [1].

Porcine LMWH from intestinal mucosa was also analyzed after digestion with a cocktail of heparinases I and II or I, II, and III (Fig. 3B; Table 3). The disaccharide content for the samples digested with only heparinases I and II ranged from 81 to 87% for IS and 13 to 19% for IIS/IIIS. Similar values were observed for IS (88%) and IIS/IIIS (12%) when the same preparation was digested with all three heparinases. No additional disaccharides were observed in digested porcine LMWH with the addition of heparinase III.

Native bovine heparin was digested with only the three-heparinase cocktail in this work (Fig. 3C). Only IS and IIS/IIIS were detected in this preparation and ranged from 85 to 86% and 14 to 15%, respectively (Table 3). With the exception of IA disaccharide, which was detected in the native porcine heparin after digestion with heparinases I, II, and III, all digested samples analyzed by direct-infusion ESI-MS had similar levels of the IS and IIS/IIIS disaccharides.

3.2. Reproducibility

Between-day reproducibility of this method was investigated with digests of native porcine heparin and porcine LMWH (Table 3; Fig. 3A and C). In this experiment, the same digest reactions were analyzed on separate days and calibrated with two different sets of calibrants (diluted from the same initial stock solutions). The between-day mean values for IS and IIS/IIIS were within 4 and 6% for heparin and LMWH, respectively, with within-day standard deviations ranging from 0.3 to 1% for heparin and 0.5 to 1% for LMWH. The results suggest there may be slight variability among the disaccharide content of these different sources of porcine heparin and LMWH. However, the combined mean values of heparin and LMWH are within 2% for both IS and IIS/IIIS, so it may be that this difference was due to experimental variability.

An additional experiment to investigate between-digest reproducibility was performed using native porcine heparin, porcine LMWH, and native bovine heparin. In this experiment, two separate sets of native porcine heparin, porcine LMWH, and native bovine heparin digest reactions were prepared and digested in parallel, and then analyzed on the same day utilizing the same set of calibrants. Similar values for disaccharides IS and IIS/IIIS were obtained in the second set of digests (Table 3). Between-set mean values were within 2% for all detected disaccharides, with standard deviation values no greater than 7%. In addition to IS and IIS/IIIS, IA was also detected, but only in the native porcine heparin samples. Otherwise, the values from this between-digest experiment were similar to those from the between-day experiment.

Porcine heparin and porcine LMWH were analyzed in both the between-day and between-digest experiments and the reproducibility between these two experiments was good (Table 3). The likelihood of heparan sulfate contamination in naturally derived heparin and LMWH preparations necessitates the inclusion of heparinase III in the enzyme cocktail to ensure that disaccharides derived from such contamination can be detected and quantified as part of the disaccharide profile of any given heparin preparation.

Although the native porcine heparin and porcine LMWH digests were stored at −20 °C between mass spectral analyses, it is possible that the disaccharide composition of these solutions may undergo changes over time. In addition, it is possible that the calibrant disaccharide content changes over time as well, which could conceal variation in the experimental samples. To avoid masking sample variability, fresh standard calibrants should be prepared from concentrated stocks before each ESI-MS analysis of digested heparin samples.

3.3. Method LOD/LOQ and linear dynamic range

LOD and LOQ values for the method ranged from 0.1 to 0.2 pmol/mg and 0.2 to 0.7 pmol/mg, respectively (Table 4). The linear range of the calibrants (1–100 pmol/mg) utilized for all calculations was above both the LOD and LOQ for this MS method. The MS^n method [17] gave a similar linear response with 1 pmol/μL as the lowest concentration analyzed for each disaccharide. LOD and LOQ values were not reported for the MS^n method. Therefore, a direct comparison of LOD and LOQ values between MS-based methods cannot be made.

3.4. Ion suppression study

As previously noted, the digests analyzed by direct-infusion ESI-MS did not undergo any type of purification before analysis.

Table 4
LOD and LOQ for individual disaccharides by direct-infusion ESI-MS

Disaccharide	*m/z*	LOD[a] (S/N = 3) (pmol/mg)	LOQ[a] (S/N = 10) (pmol/mg)
IS	288	0.1	0.2
IIS/IIIS	248	0.1	0.3
IVS	208	0.1	0.4
IA	179	0.1	0.2
IIA/IIIA	229	0.1	0.3
IVA	378	0.2	0.7

[a] Estimated by linear extrapolation of S/N values at 1 pmol/mg from calibrant mass spectra.

Therefore, the solutions that were analyzed contained multiple components, which were not present in the calibrant mixtures. To investigate whether or not the presence of additional digestion components (i.e., heparinases) had a suppressive effect on the ionization and detection of lower level disaccharides, selected heparin disaccharides at final concentrations of 1–10 pmol/mg were spiked into the methanol-quenched LMWH digest matrix before direct-infusion ESI-MS analysis. All disaccharides previously undetected in native LMWH digest reactions (IVS, IA, IIA, and IVA) were detected after being spiked in at levels greater than the LOD and LOQ (Fig. 4). Therefore, the heparin digest matrices were not suppressing the mass spectral signal of low-abundance disaccharides within the linear calibrant range.

3.5. Comparison to other methods

The relative quantitation values obtained from this ESI-MS method are compared to values obtained from other published methods of heparin disaccharide analysis in Table 5. Since the MS method cannot distinguish between the isomer pair disaccharides (IIS/IIIS and IIA/IIIA), the values from this work were reported as a sum, whereas the values from previous works were reported as those of the individual isomers. It is important to note that due to the known variation and heterogeneity of native heparins and LMWH prepared from natural sources, it cannot be assumed that the samples analyzed by various groups actually contain the same levels of disaccharides after digestion.

For native porcine heparin from intestinal mucosa (Table 5), IS was observed to be the most abundant disaccharide in heparinase digests by all methods. However, the quantitative IS values obtained from this work (84–86%) were significantly higher than those previously reported (41.2–68%). The combined IIS/IIIS values from this work (12–14%) were slightly lower than values observed from other methods, but were closer to values at the lower end of that range (16.7–37%). The IA disaccharide was found to be present at 5%, compared to values that ranged from 3.51 to 3.9%. IA was undetected by some methods. The remaining disaccharides (IVS, IIA/IIIA, and IVA) were detected at low levels by most other methods, but were not detected by the direct-infusion ESI-MS method presented here. It is interesting that in comparison to the tandem mass spectrometry method [17], which is most similar to the method employed in this paper, disaccharide IIA is not detected in our samples, whereas the other MS^n method quantitates IIA at a high proportion (30%) in unfragmented porcine heparin from the same source (Sigma). Although quantitating the proportions of the individual isomers IIA and IIIA would require MS^n, if present at such high levels, these disaccharides should have been easily detected and quantitated by the direct-infusion ESI-MS method presented in this work. It is possible that there is significant variation in the content of heparin stocks available from the same company over time, especially since it is known that different lots of the same product were analyzed here compared to that particular work (personal communication).

For native bovine heparin from intestinal mucosa (Table 5), IS was again observed to be the most abundant disaccharide in heparinase digests by all methods. In addition, the quantitative IS value obtained from this work (86%) was significantly higher than those previously reported (44.7–55.8%). The com-

Table 5
Comparison of molar percent disaccharide values in digested heparins among various methods of relative quantitative analysis

Sample source	Quantification method	IS	IIS + IIIS	IVS	IA	IIA + IIIA	IVA	Reference
Porcine heparin								
Sigma	MS[a,b]	86 ± 1.9	14 ± 1.9	ND	ND	ND	ND	This work
Sigma	MS[a,b]	84 ± 4.1	12 ± 4.2	ND	5 ± 0.5	ND	ND	This work
Sigma	MS^n [d]	41.2 ± 2.0	16.4 ± 2.4 + 4.5 ± 0.7	1.7 ± 0.5	2.9 ± 0.9	30.0 ± 4.2 + 0.7 ± 0.1	2.6 ± 0.8	[17]
Pharmacia	CE-UV	68	12 + 4.7	1.2	3.9	2.5 + 1.5	0.2	[13]
Bioiberica	CE-UV	65.8	13.5 + 7.5	2.63	3.51	41.14 + 1.18	1.68	[12]
Seikagaku Corp.	HPLC-UV	59.8	20.3 + 7.6	3.5	ND	3.4 + ND	5.4	[14]
Seikagaku Corp.	HPLC-fluorescence	49.6	29.8 + 7.2	7.6	ND	2.3 + ND	3.5	[14]
Bovine heparin								
Sigma	MS[a,c]	86 ± 4.2	15 ± 4.2	ND	ND	ND	ND	This work
Sigma	MS^n [d]	44.7 ± 2.7	10.5 ± 0.4 + 19.4 ± 0.7	3.7 ± 0.3	0.6 ± 0.4	12.8 ± 2.4 + 4.7 ± 0.9	3.5 ± 0.3	[17]
Purified in lab	HPLC-UV[e]	55.8	12.5 + 19.2	3.8	NR	2.4 + NR	6.3	[16]
Bioiberica	CE-UV	55.4	8.9 + 30.5	2.74	0.51	0.24 + 1.53	0.23	[12]
Porcine LMWH								
Sigma	MS[a,b]	84 ± 0.9	16 ± 0.9	ND	ND	ND	ND	This work
Sigma	MS[a,c]	88 ± 3.2	12 ± 3.2	ND	ND	ND	ND	This work
Sigma	CE-UV	80.4	7.3 + 4.55	0.60	5.86	1.03 + 0.28	ND	[12]

ND: not detected; NR: not reported.
[a] Mean ± standard error of the mean from two sets.
[b] Digested with heparinases I and II.
[c] Digested with heparinases I, II, and III.
[d] Mean standard deviation from one set.
[e] Values reported as mass%.

bined IIS/IIIS values from this work (15%) were significantly lower than values observed from other methods (29.9–39.4%). No other disaccharides were detected by the MS method presented here, while some were detected at low levels by several of the other methods. Similar to native porcine heparin, IIA/IIIA were detected at significantly high levels (17.5%) [17], but remained undetected by the MS method. Again, the native bovine heparin stocks analyzed by both the MS method in this work and the MS^n method [17] were supplied from the same source (Sigma), but significant variation may exist between the lots.

Since fewer analysis techniques have been used to determine the disaccharide content of porcine LMWH, the values obtained by MS are compared to only one other method in Table 5. The values obtained for IS and IIS/IIIS by MS are similar to those of the CE-UV method (84–88% versus 80.4% and 12–16% versus 11.85%, respectively). No other disaccharides were detected in porcine LMWH by MS, while IVS, IA, and IIA/IIIA were detected at low levels (0.60–5.86%) by CE-UV. It is unknown if the porcine LMWH analyzed by these two methods was from the same lot, though given the amount of time that has passed between studies, it would seem unlikely. However, it is possible that the processing of LMWH from native heparin may result in a final disaccharide content that is more reproducible from lot to lot.

Overall, IS was detected as the most abundant disaccharide in all samples analyzed by MS, resulting in quantitative values often significantly higher than those reported by other methods for similar samples. IIS/IIIS disaccharides were the second most abundant in all samples, with quantitative values lower than those obtained through other methods. Other methods often detected additional disaccharides at low levels that were not detected by MS. While it can be helpful to compare the results from various published methods, it would be necessary for one group to measure the same samples on multiple platforms or multiple groups to participate in a round-robin study analyzing the same samples by multiple methods, to validly compare the results of these various quantitative methods of heparin disaccharide determination.

In general, all of these methods for analyzing heparin disaccharide content required digestion with heparinases. Therefore, this sample processing step was relatively equal among methods. Heparin disaccharide analysis methods based on MS, such as the work presented here, and MS^n methods [17,18] offer the dual benefits of straightforward sample preparation and rapid analysis, since sample purification and separation by CE or HPLC can add significant amounts of time to the analysis. Several of the methods compared here employ UV absorbance detection of disaccharides at 232 nm [12,16]. To improve the detection sensitivity, other groups employed a fluorophore-derivatization step [13,14]. However, such a step adds time and variability to sample preparation.

It is noted that all methods of analyzing heparin disaccharide content thus far provide relative quantitative results of known disaccharides. An additional possibility may be to integrate stable-isotope labeled internal standard disaccharides into various heparin samples before digestion, which could allow for absolute quantitation of disaccharides of choice by isotope-dilution mass spectrometric analysis.

4. Conclusions

The ESI-MS method presented here appears promising as a means to identify and quantitate the relative levels of known disaccharides in various heparin and LMWH preparations. The information derived from this method of analysis could assist in the further characterization of heterogeneous preparations of heparin and LMWH pharmaceuticals, by not only quantitating the disaccharide content native to heparin, but also in identifying levels of possible heparan sulfate contamination based on the levels of disaccharides released by heparinase III digestion. After 24 h of digestion, samples are ready for direct analysis without further purification or separation. However, the direct-infusion technique currently employed to introduce the samples into the mass spectrometer does not lend itself to an automated method, since exchanging samples requires the manual loading and replacing of a syringe into a syringe pump. Automated injection of calibrant mixes and samples or the creation of a complementary quantitative LC/MS/MS method could help to resolve this issue, as well as provide for the possible separation of disaccharide isomer pairs prior to MS^n detection.

Mass spectral analysis of heparin disaccharides is sensitive and selective, insuring that disaccharides with end-groups altered by initial depolymerization processing are not included in the quantitative determinations of the known disaccharide standards. The future availability of end-group altered disaccharide standards may allow for the quantitation of additional disaccharides formed under the various processing conditions or those that may be present in synthetic heparins or LMWHs.

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