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Characterization of Currently Marketed Heparin Products: Reversed-Phase Ion-Pairing Liquid Chromatography Mass Spectrometry of Heparin Digests

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Here we report results from the analyses by enzymatic digestion and reversed-phase ion-pairing liquid chromatography mass spectrometry (RPIP-LC-MS) of active pharmaceutical ingredient (API) unfractionated heparins (UFHs) from six different manufacturers and one USP standard sample. We employed a reverse phase ion-pairing chromatography method using a C_{18} column and hexylamine as the ion-pairing reagent with acetonitrile gradient elution to separate disaccharides generated from the digestion of the heparins by lyase I and III (E.C. 4.2.2.7 and 4.2.2.8) before introduction into an ion-trap mass spectrometer by an electrospray ionization (ESI) interface. Extracted ion chromatograms (EICs) were used to determine the relative abundance of the disaccharides by mass spectrometry. Eight disaccharides were observed and a similar composition profile was observed from digests of 20 UFH samples. The compositional profile determined from these experiments provides a measure of the norm and range of variation in "good" heparin to which future preparations can be compared. Furthermore, the profile obtained in the RPIP-LC-MS assay is sensitive to the presence of the contaminant, oversulfated chondroitin sulfate A (OSCS), in heparin.

Heparin is a highly sulfated, long chain polysaccharide with an average molecular weight of 15 kDa (mass range 5−40 kDa), that consists of alternating uronic acid residues (1 → 4) linked to glucosamine.[1] Complexity is introduced into the oligosaccharide chain during biosynthesis by the action of epimerases and sulfotransferases.[2] The uronic acid residues may be *O*-sulfated at the C2 position, while the glucosamine may be *O*-sulfated at the C6 position, as well as the C3 position, the latter being rare but important for the binding of heparin to antithrombin III.[3] The nitrogen of the glucosamine residue can exist as a free amine, an *N*-acetylated amine, or an *N*-sulfated amine. Upon digestion with a cocktail of heparin lyases, this combination of modifications results in the possibility of 12 distinct disaccharide units (see Table 1), ignoring the rare C3 O-sulfated disaccharides.

As a pharmaceutical agent, heparin is widely used as an anticoagulant for the prevention of the formation of blood clots during and after surgery, as well as in dialysis patients.[4−6] The biological action of heparin is complex and is not limited to its anticoagulant activity alone, for example the use of heparin has been investigated as a treatment for senile dementia[7,8] and cancer.[9] Heparin is isolated from animal sources, most commonly the mast cells found in porcine intestinal mucosa. This raw material undergoes extensive physical and chemical clean up prior to coming to market. Generally, unfractionated heparin (UFH) is administered intravenously and has a short half-life and concomitantly must be constantly infused or administered frequently.

The purification and sale of heparin is a multibillion dollar a year industry, so there is an economic motivation to attempt to counterfeit or adulterate heparin for monetary gain. This was recently manifested with tragic consequences in 2008 with the contamination of heparin with oversulfated chondroitin sulfate A (OSCS).[10,11] OSCS is a synthetic material that is made by oversulfation of chondroitin sulfate A (CSA), which is a native glycosaminoglycan (GAG) that can copurify with heparin. The presence of OSCS in heparin went undetected by the United States

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Table 1. Molecular Weights, Observed *m/z* Values, Retention Times, Ionized Species, And Response Factors for the Eight Experimentally Observed Disaccharides Samples

disaccharide	MW (g/mol)	Obs. *m/z*	RT (Min)	ionized species	response factor
I-S	577.0	982.0	34.9	$(M - 3H + 4HA)^+$	$y = 0.942x + 0.1164$
II-S	497.0	801.0, 901.4	30.8	$(M - 2H + 3HA)^+$, $(M - 3H + 4HA)^+$	$y = 0.704x + 0.0059$
III-S	497.0	801.0, 901.4	31.0	$(M - 2H + 3HA)^+$, $(M - 3H + 4HA)^+$	$y = 0.682x + 0.0562$
IV-S	417.1	620.1	25.3	$(M - H + 2HA)^+$	$y = 0.684x + 0.0069$
I-A	539.0	843.3	31.5	$(M - 2H + 3HA)^+$	$y = 0.817x + 0.0220$
II-A	459.1	662.2	25.5	$(M - H + 2HA)^+$	$y = 0.614x + 0.0022$
III-A	459.1	662.2	25.6	$(M - H + 2HA)^+$	$y = 0.670x - 0.0021$
IV-A	379.1	481.1	~11−15	$(M + HA)^+$	$y = 0.347x + 0.0015$
I-H	497.0	801.0	25.7	$(M - 2H + 3HA)^+$	$y = 0.291x + 0.0219$
II-H	417.1	620.1	~9−12	$(M - H + 2HA)^+$	$y = 0.448x + 0.0003$
III-H	417.1	620.1	~9−12	$(M - H + 2HA)^+$	$y = 0.266x + 0.0055$
IV-H	337.1	439.0	3.1	$(M + HA)^+$	$y = 0.076x + 0.0102$
I-P	553.0	857.2	32.4	$(M - 2H + 3HA)^+$	$y = x$

Pharmacopeia (USP) testing methods which did not measure the composition or structure of heparin at that time. Administration of contaminated heparin to patients lead to 574 reports of adverse reactions and 94 deaths[12] in the U.S. and abroad. Prior to the contamination crisis of 2008, the structural characterization of heparin was not deemed necessary to assess drug quality, thus there was a range of variation in the content of copurified impurities in heparin.[13]

Reverse phase ion-pairing (RPIP) chromatography with ion-pairing reagents has been applied to the separation of heparin disaccharides to improve their retention on reverse phase and to neutralize charge for introduction to mass spectrometers for analysis. Doneanu et al. did extensive work investigating the effect the identity and concentration of the ion pairing reagent had on the performance of the chromatographic separation and liquid chromatography mass spectrometry (LC-MS) analysis.[14] They applied this information to the analysis of large heparin derived oligosaccharides and intact LMWH. Jones et al. used the structural differences inherent in heparin disaccharides to study the mechanism of separation in RPIP-LC.[15] By evaluating the changes in retention time and performance of standard disaccharides using different ion pairing reagents and experimental conditions they proposed that there are two mechanisms of separation: electrostatic interactions of the positively charged ion pairing reagent imbedded in the stationary phase with the analyte or hydrophobic interaction of preformed ion pair/analyte complexes with the C_{18} stationary phase. The extent to which mechanism is operating is dependent on experimental conditions.

Mass spectrometric analysis of heparin and heparin derived disaccharides has been performed with and without separation prior to analysis. Direct infusion by electrospray ionization (ESI) allows for rapid screening and minimal sample handling.[16] Quantification is accomplished by applying predetermined response factors to the observed areas of the peaks of interest. Tandem MS and MS^n can be used to differentiate structural isomers, such as II-A and III-A, if desired.[17,18] Separation prior to analysis helps to avoid some of the problems associated with direct infusion methods, such as the possibility of ion suppression. Capillary electrophoresis has been coupled to MS to provide separation prior to analysis.[19,20] RPIP-HPLC provides good separation of most heparin disaccharides prior to analysis, but is often time-consuming. However, higher pressure HPLC methods (aka UPLC) have been developed which provide baseline separation for 10 of the 12 known disaccharides in as little as five minutes.[21]

The work described here was undertaken to establish what could be considered a baseline value for the disaccharide composition of safe and effective heparin utilizing equipment available in many analytical laboratories. We analyze 20 UFH samples obtained from six manufacturers. For five of the manufacturers we tested multiple lots so a measure of the lot-to-lot variation by a single manufacturing process could be assessed. We find the compositional profiles obtained by RPIP-LC-MS analysis are largely invariant across all of manufacturing processes assessed.

EXPERIMENTAL SECTION

Reagents. Fisher Optima LC/MS grade acetonitrile (ACN) and water were used in making up LC solvents. Hexylamine, from Acros Organics, was used as the ion pairing reagent. Formic acid, 99+%, was purchased from Thermo Scientific. Heparin disaccharide standards were purchased from Sigma Aldrich (St Louis, MO). UFHs representing lots produced from May 2007 to June 2009, all originating from porcine intestinal mucosa, were obtained by the FDA from multiple manufacturers. Enzymes, EC 4.2.2.7 and EC 4.2.2.8, were purchased from Associates of Cape Cod, Inc. (East Falmouth, MA).

Heparins and Heparin Digests. UFH APIs were provided to the FDA from six different manufacturers. Prior to digestion the heparin samples were desalted by pressure dialysis with more

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than one liter of 18 MΩ water using an Amicon 8200 stirred cell with a 1000 molecular weight cutoff filter. The desalted heparin solutions were lyophilized to dryness using a Millrock benchtop freeze-dryer in preparation for enzymatic digestion.

Enzymatic digestions were carried out on 40 μg of heparin using 10 milli-IU each of EC 4.2.2.7 (lyase I, heparinase) and EC 4.2.2.8 (lyase III, heparitinase). A single International Unit (IU) of enzyme is capable of producing 1 μmole of unsaturated uronic acid per minute at 37 °C, pH 7.0. Eliminative cleavage by the enzyme results in the formation of a double bond between C4 and C5 of the uronic acid on the nonreducing end of the oligosaccharide. The total reaction volume of 100 μL consisted primarily of 20 mM ammonium acetate and 2 mM calcium acetate (pH 7.4). The reaction solution was incubated for 24 h at 37 °C in a water bath. The digestion was stopped by separating the enzyme from the digestion products using a centrifugal filter with a 3k molecular weight cutoff (Amicon Ultra-0.5 purchased from Millipore, Billerica, MA). Each sample was washed through the membrane with two additional 100 mL aliquots of 18 MΩ water. The flow through, which contained the digestion products, was retained for LC-MS analysis.

The extent of the digestion was verified by PAGE analysis of the retentate, no bands corresponding to large oligosaccharides were observed. Another check of the completeness of the digestion was made by extending the RPIP-LC-MS gradient to 75% ACN and monitoring the chromatogram. Fondaparinux (10 negative charge sites, 8 sulfates and 2 carboxylates) served as a model for hexa- and octasacchrides. Fondaparinux eluted in approximately 50% ACN as a doubly charged ion paired species at m/z 1260.1. When this gradient profile was used to analyze one of the enzyme digest solutions, no signal corresponding to oligosaccharides larger than a disaccharide was observed. For this reason and to reduce the amount of contamination introduced into the mass spectrometer, the final gradient composition was set to 45% ACN. A final check on the completeness of the digestion was done by adding a second aliquot of the enzymes to the reaction mixture after 24 h, and then allowing the digesting to run for another 24 h. The analysis of the digestion products that resulted showed no differences from that obtained with one aliquot of enzyme and 24 h digestion.

RPIP-LC-MS. LC separations were carried out using an Agilent 1100 series LC system, consisting of a degasser, quaternary solvent pump, thermostatted column compartment, and autosampler. The samples were separated at a flow rate of 150 μL/min using a Waters XBridge C_{18} column with 3.5 μm particles and dimensions of 2.1 × 150 mm. The column compartment was heated to 40 °C during analysis. Solvent A consisted of 15 mM hexylamine in 100% water, pH ~ 7 (pH was adjusted using formic acid and measured using Whatman pH paper). Solvent B consisted of 15 mM hexylamine in 75% ACN, apparent pH ~ 7. The solvent composition was held at 0% B for the first five minutes, than increased to 60% B over 35 min, where it was held for five minutes, finally it was returned to 0% B for the last 10 min of the run. During the final 10 min of the run, the valve on the mass spectrometer was switched to waste in order to reduce the amount of contamination in the source. Re-equilibration of the column with 10 min of 0% B was necessary at the end of the run to ensure consistent retention of the analytes from run to run.

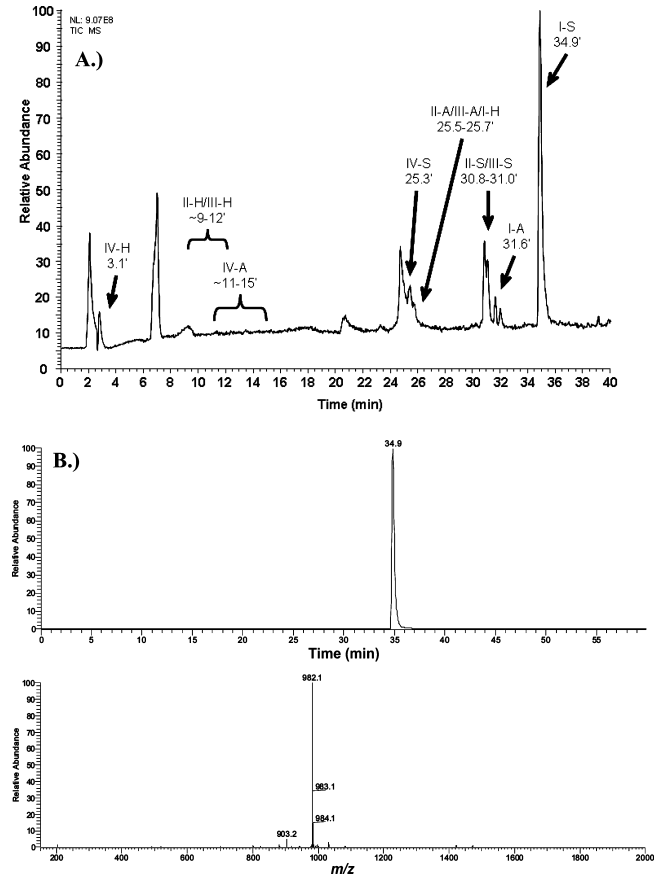
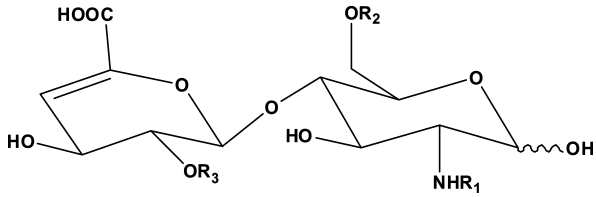


Figure 1. Panel A shows the TIC from the injection of a digest of one of the heparin samples. The locations of the elution positions of disaccharide standards are noted. The presence of a ~ symbol indicates a broad peak. Disaccharide composition was measured based on the area of the peak obtained from extracted ion current (EIC) spectra for the respective disaccharides as shown in Panel B for the EIC of m/z 982, corresponding to the disaccharide I-S eluting at 34.9' in Panel A. The mass spectrum shows the disaccharide I-S associated with four hexylamine ions (one for each negatively charged sulfate group) and one proton, resulting in a net charge of +1. Despite the fact that I-S contains three sulfate groups, the signal due to the loss of sulfate is negligible. The small peak at m/z 903 results from the replacement of one hexylamine cation with one sodium cation (m/z 102 vs 23, respectively).

Mass analysis was carried out by directly connecting the LC effluent line to the ESI source on a Thermo LCQ Deca XP. The use of an ion pairing reagent allowed the MS analysis to be performed in the positive mode, which others have shown to produce approximately twice the signal than that observed in the negative mode in this type of experiment.[14] The spray voltage was set to 4.5 kV, the sheath gas at 60 (arbitrary units), and the capillary temperature at 275 °C. Prior to analysis, the instrument was tuned using resperpine (m/z 609). The instrument was set up to scan from 150−2000 amu in the full MS mode throughout the entire LC run. The disaccharides were quantified using peak area from the extracted ion chromatogram (EIC) of the ion of interest at the appropriate retention time. The EICs were produced by searching a m/z window that was ±0.5 mass units from the ion of interest. A representative TIC for a digest and an EIC and mass spectrum for I-S are shown in Figure 1. The presence of



Disaccharide	R_1	R_2	R_3
I-S	SO_3^-	SO_3^-	SO_3^-
II-S	SO_3^-	SO_3^-	H
III-S	SO_3^-	H	SO_3^-
IV-S	SO_3^-	H	H
I-A	Ac	SO_3^-	SO_3^-
II-A	Ac	SO_3^-	H
III-A	Ac	H	SO_3^-
IV-A	Ac	H	H
I-H	H	SO_3^-	SO_3^-
II-H	H	SO_3^-	H
III-H	H	H	SO_3^-
IV-H	H	H	H

Figure 2. The structures of the 12 commercially available disaccharides that result from enzymatic depolymerization of heparin and heparan sulfate with a combination of heparin lyases.

the hexylamine ion pairing reagent stabilizes the sulfate groups and results in negligible amounts of sulfate loss.[14]

RESULTS

Disaccharide Standards. Twelve disaccharide standards, products resulting from the enzymatic depolymerization of heparin and heparan sulfate by various heparinases, were purchased from Sigma. These disaccharides are primarily classified based on the substituent present on the nitrogen of the glucosamine. If the disaccharide contains a free amino group, it is given the designation, H, an acetylated amino group, A, or a sulfated amino group, S. The disaccharides within each of these groups are further classified based on the degree of sulfation. The most highly sulfated disaccharide within a particular group is labeled with the Roman numeral I, the next most highly sulfated II, and so on. To illustrate this naming convention, the most highly N-sulfated disaccharide is called I-S. If two disaccharides within the same group have an equivalent number of sulfates, the one with the greater number of sulfates on the glucosamine ring is given the lower Roman numeral. Structures of the 12 disaccharides can be derived from the information given in Figure 2. The concentrations of the disaccharide stock standards were determined using UV−vis measurements at 232 nm, with a molar extinction coefficient of 5500 cm^{-1} M^{-1}.[22] The concentrations calculated by UV absorption ranged from 50 to 150% of the expected value calculated using the amount listed on the vial by the manufacturer.

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RPIP LC-MS of Heparin Digests. A series of five solutions with disaccharide concentrations ranging from 5×10^{-9} to 1×10^{-4} M were made and analyzed using the same experimental conditions as those used for the enzymatic digests of heparin. This allowed for the determination of retention times, observed molecular masses, and response factors (see Table 1). Although the disaccharide solutions were made up over a 5 orders of magnitude concentration range, the linear range of detection was approximately 3 orders of magnitude. The retention time on the C_{18} column was directly related to the number of hexylamine molecules associated with a particular disaccharide, which in turn was directly related to the number of sulfate groups in the disaccharide, plus one hexylamine for the carboxylate group of the uronic acid. *N*-acetylated disaccharides elute at slightly longer times than other disaccharides with the same number of sulfate groups. The position of sulfation also effects the retention time. Disaccharides sulfated on the C6 position of the glucosamine elute at slightly shorter times than those sulfated at the C2 position of the uronic acid. The H series of disaccharides eluted as broad doublets, presumably due to the partial resolution of α-and β-anomers that result from mutarotation of the glucosamine residue.[23]

Using the current chromatography method we were unable to obtain full baseline separation of II-S from III-S, II-A from III-A, and II-H from III-H. Since the members of each pair are isomers, the MS was also unable to separate the two disaccharides using an extracted ion chromatogram. MS^n would enable separation of the two disaccharide pairs based on their unique fragmentation patterns, but this technique was not employed here because our focus was on development of a robust method for compositional profiling of heparin. Since we were not able to separate the isomers by the techniques employed, the areas of each pair were summed and the results presented as the total amount of II-S/III-S, II-A/III-A, or II-H/III-H.

Disaccharide Composition of Market Place Heparins. RPIP-LC-MS analysis of the disaccharides produced by exhaustive enzymatic digestion of UFHs revealed that the major disaccharide unit was I-S, comprising approximately 65 ± 2% of the oligosaccharide chain. The results of 20 different lots of unfractionated heparin from six different manufacturers are summarized in Figure 3. Overall the composition between lots and between manufacturers is consistent with a %RSD of 3.1%. Glucosaminoglycans from different species[20,24−26] or isolated from different organs[21,26] have varying disaccharide compositions. However, all of the samples analyzed here originated from porcine intestinal mucosa. Of the 12 common disaccharides only eight were observed at detectable levels in the chromatogram of the digest. The series of disaccharides containing a free amine on the glucosamine residue were not observed in these experiments. Importantly, we did observe amine containing disaccharide

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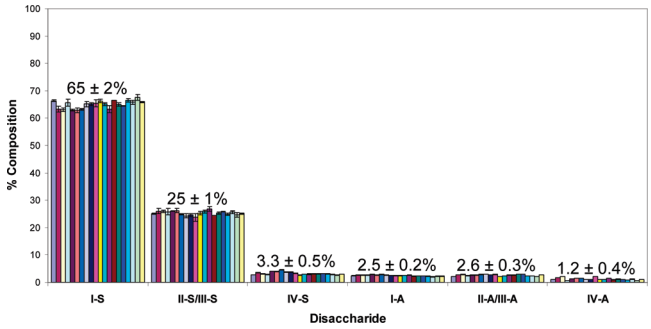

Figure 3. The relative disaccharide composition of 20 different lots of heparin from seven different sources by RPIP LC-MS. The disaccharides resulted from exhaustive enzymatic digestion of heparin using a mixture of heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8). The results shown are the averages and standard deviations from three injections of one digestion for each lot. Values above each disaccharide category are the mean and standard deviations over all lots.

standards (Table 1) and have performed similar analysis of heparan sulfate digests where free amine containing disaccharides were observed (data not shown).

The less common, but biologically important, disaccharide ΔUA2S→GlcNS3S is isomeric with I-S and therefore was not distinguished from I-S in the experiments described here. Others have demonstrated that the two species can be differentiated based on the fragmentation patterns observed when using tandem MS and MS^3 experiments,[27] so relative abundance of this rare disaccharide could be determined using RPIP-LC-MS by this approach. Our interest in this work was on the composition of the major disaccharides present in the digests to establish a fingerprint amenable to identification of outliers.

The disaccharide compositions obtained in our experiments are in good agreement with percent composition values obtained by others with CE-fluorescence and HPLC (see Table 2). The average percentage composition of I-S from the six examples listed in Table 2 is 66 ± 10%, with a range of values from 53.6−84%. If the methods of direct infusion ESI MS are removed from the comparison the mean is 65 ± 3% with a range of 59.8−68.0%. A direct infusion approach offers the advantages of rapid analysis and reduced equipment requirements, but it may not result in a reliable measure of the composition due to ion suppression effects or differential cationization.

Inhibition by Oversulfated Chondroitin Sulfate. Oversulfated chondroitin sulfate (OSCS) is known to inhibit the action of heparin lyases.[28,29] To investigate the effect the presence of OSCS has on this assay, several standards were made up by spiking OSCS into a solution of heparin. The amount of OSCS spiked into the heparin solution was 0.1, 0.5, 1, 2, 5, and 10% by weight. These solutions were then subjected to the same digestion and RPIP-LC-MS procedure described earlier.

In our experiments, the inhibition of the heparin lyases by OSCS manifested itself in two ways: (1) the observed percentage of I-S increased significantly and (2) the overall intensity of the spectrum, with respect to the disaccharide signals, decreased, despite the fact that the same experimental conditions were employed. We attribute this increase in I-S percentage to the fact

Table 2. Disaccharide Composition of Market Place Heparins (Top) Isolated from Porcine Intestinal Mucosa and Analyzed by a Variety of Techniques (HPLC, CE, ESI-MS and HPLC MS)[a]

UFH disaccharide	I-S	II-S/III-S	IV-S	I-A	II-A/III-A	IV-A	I-H	II-H/III-H	IV-H	Method
this work	65 ± 2	25 ± 1	3.3 ± 0.5	2.5 ± 0.2	2.6 ± 0.3	1.2 ± 0.4	ND	ND	ND	RPIP LC-MS
Saad et al.[18] (Sigma)	53.6 ± 1.7	15.5 ± 1.4	7.8 ± 2.7	2.7 ± 0.2	5.4 ± 0.8	7.2 ± 1.9	ND	0.7 ± 0.3	6.0 ± 0.4	Direct Infusion ESI MS and MS/MS
Kinoshita and Sugahara[30] (Seikagaku Corp.)	59.8	27.9	3.5	ND	3.4	5.3	ND	ND	ND	HPLC UV detection
Camara et al.[16] (Sigma)	84 ± 4.1	12 ± 4.2	ND	5 ± 0.5	ND	ND	ND	ND	ND	Direct Infusion ESI MS
Ruiz-Calero et al.[26] (Bioiberica)	65.8	21.0	2.63	3.51	5.32	1.68	ND	ND	ND	CE
Militsopoulou et al.[31] (Pharmacia)	68.0	16.7	1.2	3.9	4	0.2	2.35	3.5	0.15	CE

[a] ND indicates that a particular disaccharide was not detected in the analysis.

Table 3. Relative Disaccharide Composition of Heparin Spiked with Various Levels of Synthetic OSCS or Authentically Contaminated Samples from Triplicate Injections of a Single Digestion[a]

sample	I-S[b]	II-S/III-S	IV-S	I-A	II-A/III-A	IV-A
MP#1	65.6 ± 0.2	25.6 ± 0.2	2.7 ± 0.1	2.6 ± 0.1	2.3 ± 0.1	1.2 ± 0.0
MP#1 + 0.1% OSCS	67.1 ± 0.3	27.5 ± 0.4	3.0 ± 0.2	1.4 ± 0.0	0.8 ± 0.0	0.2 ± 0.0
MP#1 + 0.5% OSCS	73.3 ± 0.4	22.7 ± 0.6	2.1 ± 0.0	1.0 ± 0.0	0.6 ± 0.0	0.3 ± 0.0
MP#1 + 1.0% OSCS	82.0 ± 0.9	15.2 ± 1.1	2.0 ± 0.2	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
MP#1 + 2.0% OSCS	82.8 ± 0.1	9.9 ± 0.1	2.0 ± 0.2	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
MP#1 + 5.0% OSCS	94.6 ± 0.4	3.3 ± 0.6	2.0 ± 0.3	ND	ND	ND
MP#1 + 10.0% OSCS	100 ± 0	ND[c]	ND	ND	ND	ND
Heparin average[d]	65 ± 2	25 ± 1	3.3 ± 0.5	2.5 ± 0.2	2.6 ± 0.3	1.2 ± 0.4
sample A (0.5% OSCS)	69.4 ± 0.3	25.6 ± 0.5	2.7 ± 0.2	1.3 ± 0.0	0.7 ± 0.0	0.3 ± 0.0
sample B (1.3% OSCS)	81.1 ± 0.1	15.8 ± 0.1	2.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.1	0.2 ± 0.0
sample C (2.1% OSCS)	81.1 ± 0.4	14.7 ± 0.6	3.2 ± 0.3	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
sample D (5.9% OSCS)	94.6 ± 0.4	3.3 ± 0.6	2.0 ± 0.3	ND	ND	ND
sample E (10.0% OSCS)	100 ± 0	ND	ND	ND	ND	ND

[a] The top seven digests are from a single heparin sample (MP#1) spiked with synthetic OSCS. The bottom five digests (Samples A−E) are authentically contaminated heparin APIs whose OSCS levels were measured by SAX-HPLC. [b] The *P* values calculated when comparing the mean of each of the OSCS contaminated samples to that of MP#1 were all less than 0.005. [c] ND indicates not detected. [d] Average values obtained over all twenty marketplace heparin samples Table 2.

that the majority of the heparin chain is composed of I-S; therefore, a cut made by the enzyme is more likely to release I-S than any other disaccharide. For this reason, while I-S is still detectable in the spectrum, the intensity from the other disaccharides is lost or concealed by the background due to enzyme inhibition in the presence of OSCS. Since we are reporting relative percent compositions, if signal is lost from the minor disaccharide components, the observed percentages of those disaccharides that remain, namely I-S, increase (Table 3). At a level of between 5 and 10% OSCS, the analysis begins to show I-S as the only disaccharide component (Table 3). At lower contamination levels significant effects on the disaccharide composition relative to control digests are observed at the 0.1% OSCS level.

If careful attention is paid to the area representing the amount of the disaccharide present, one will see that as the amount of OSCS contamination increases, the absolute measure of the area decreases. In our experiments the area under the curve representing I-S was decreased by 60% when the sample was contaminated with 2% OSCS by weight. At the same time, the area corresponding to the internal standard I-P was unaffected, so the issue was not related to a decrease in the sensitivity of the instrument. Thus, the observed signal decreases because less disaccharide is being produced due to inhibition of the enzymes by OSCS and because longer heparin chains are not observed with the method applied here.

Five authentically OSCS contaminated samples obtained by the FDA were analyzed using RPIP-LC-MS (Table 3). These samples were contaminated with 0.5, 1.3, 2.1, 5.9, and 10.0% OSCS by weight as determined by strong anion exchange HPLC. Results of the RPIP-LC-MS analysis revealed a disaccharide composition which was outside the norms established here from digestion of unadulterated heparin samples. The disaccharide peak areas also decreased relative to the internal standard, indicating diminished enzymatic efficiency.

CONCLUSIONS

In the aftermath of the recent heparin contamination crisis, many researchers have worked to develop better methods by which to rapidly and thoroughly screen API heparins in order to prevent a reoccurrence of such an event. Here we present an RPIP-LC-MS approach to determine what can be considered the normal disaccharide composition of quality UFH. We have found that the disaccharide composition of heparin derived from porcine intestinal mucosa is consistent from one manufacturer to the next and across lots. Deviation from the composition pattern can be used as an indicator of altered composition or potential adulteration.

In looking forward, the use of UPLC based methods promise better separation of heparin derived disaccharides on a much shorter time scale.[21] Coupled with a high performance rapid scanning MS, like a qTOF, a more complete analysis of the disaccharide composition of heparin is possible. Chromatographic separation of the isomeric disaccharide pairs and increased sensitivity will undoubtedly provide a clearer picture of heparin's composition.

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