Analysis of Pharmaceutical Heparins and Potential Contaminants Using ^{1}H-NMR and PAGE

ZHENQING ZHANG,[1,2] BOYANGZI LI,[1,2] JIRAPORN SUWAN,[2,3] FUMING ZHANG,[2,4] ZHENYU WANG,[2,5] HAIYING LIU,[1,2] BARBARA MULLOY,[6] ROBERT J. LINHARDT[1,2,4,5]

[1]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, New York 12180

[2]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, New York 12180

[3]Faculty of Medicine, Department of Biochemistry, Thailand Excellence Center for Tissue Engineering, Chiang Mai University, Chiang Mai 50200, Thailand

[4]Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, New York 12180

[5]Department of Biology, Rensselaer Polytechnic Institute, Troy, New York 12180

[6]National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts EN6 3QG, UK

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ABSTRACT: In 2008, heparin (active pharmaceutical ingredient, API) lots were associated with anaphylactoid-type reactions. Oversulfated chondroitin sulfate (OSCS), a semi-synthetic glycosaminoglycan (GAG), was identified as a contaminant and dermatan sulfate (DS) as an impurity. While DS has no known toxicity, OSCS was toxic leading to patient deaths. Heparins, prepared before these adverse reactions, needed to be screened for impurities and contaminants. Heparins were analyzed using high-field ^{1}H-NMR spectroscopy. Heparinoids were mixed with a pure heparin and analyzed by ^{1}H-NMR to assess the utility of ^{1}H-NMR for screening heparin adulterants. Sensitivity of heparinoids to deaminative cleavage, a method widely used to depolymerize heparin, was evaluated with polyacrylamide gel electrophoresis to detect impurities and contaminants, giving limits of detection (LOD) ranging from 0.1% to 5%. Most pharmaceutical heparins prepared between 1941 and 2008 showed no impurities or contaminants. Some contained DS, CS, and sodium acetate impurities. Heparin prepared in 2008 contained OSCS contaminant. Heparin adulterated with heparinoids showed additional peaks in their high-field ^{1}H-NMR spectra, clearly supporting NMR for monitoring of heparin API with an LOD of 0.5–10%. Most of these heparinoids were stable to nitrous acid treatment suggesting its utility for evaluating impurities and contaminants in heparin API. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:4017–4026, 2009

Keywords: heparin; contamination; ^{1}H-NMR; heparinoids; nitrous acid

INTRODUCTION

Heparin is a polydisperse mixture of linear acidic polysaccharides and has been in clinical use as an anticoagulant over 75 years. [1–3] Heparin is unique as one of the oldest drugs currently still in widespread clinical use, one of the first biopolymeric drugs, and one of the few carbohydrate

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Correspondence to: Robert J. Linhardt (Telephone: 518-276-3404; Fax: 518-276-3405; E-mail: linhar@rpi.edu)

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drugs.[3] Heparin is composed of a repeating disaccharide structure of 1,4-linked hexuronic acid and glucosamine residues (Fig. 1A). The most common disaccharide unit of heparin is composed of a 2-*O*-sulfo-α-L-iduronic acid 1,4 linked to 6-*O*-sulfo-*N*-sulfo-α-D-glucosamine, -IdoA2S (1,4)GlcNS6S-.[4] Heparin is isolated by extraction from animal tissues, most commonly porcine intestine, and is a member of the glycosaminoglycan (GAG) family.[5] Recently, a new, rapid onset, acute side effect associated with heparin was observed, believed to be caused by an anaphylactoid response.[6] This spike in adverse events was associated with an oversulfated chondroitin sulfate (OSCS) contaminant (Fig. 1D), semi-synthesized from another GAG, chondroitin sulfate (CS, Fig. 1B), in certain lots of heparin.[7,8] Since heparin has not routinely been analyzed by modern spectroscopic methods, there has been some concern that past lots of pharmaceutical heparins might also have contained contaminants or impurities. An impurity is a substance that can be introduced or retained in the natural processing of heparin from animal tissue while a contaminant is a substance that is accidentally or deliberately added outside of a normal process step. Dermatan sulfate (DS or CS-B, Fig. 1C) has been previously identified as an impurity in pharmaceutical heparin.[9] In addition to OSCS, dozens of natural and artificial heparinoids discovered and studied over the past 30 years have charge densities, molecular weights, and anticoagulant activities similar to those of heparin.[10] It is unclear whether any of these heparinoids found their way into heparin API in the past, moreover, the risk of future contamination of heparin still exits. New, more effective and convenient analytical methods are now needed to monitor the quality of heparin API and formulated drug to detect impurities and contaminants. High-field ^{1}H-NMR is widely used by chemists to analyze the structures of heparin and heparinoids.[11–21] During the recent contamination crisis, ^{1}H-NMR was critical in identifying the structure of OSCS contaminating heparin.[7] ^{1}H-NMR is also useful for the quantitative determination of OSCS and DS in heparin.[22] Other methods including selective chemical and enzymatic depolymerization were also useful in demonstrating the presence of OSCS contaminant in heparin and these methods did not require specialized and expensive instrumentation.[23] After chemical or enzymatic depolymerization of heparin, resistant DS and OSCS remained intact and could be detected using electrophoresis or chromatography.

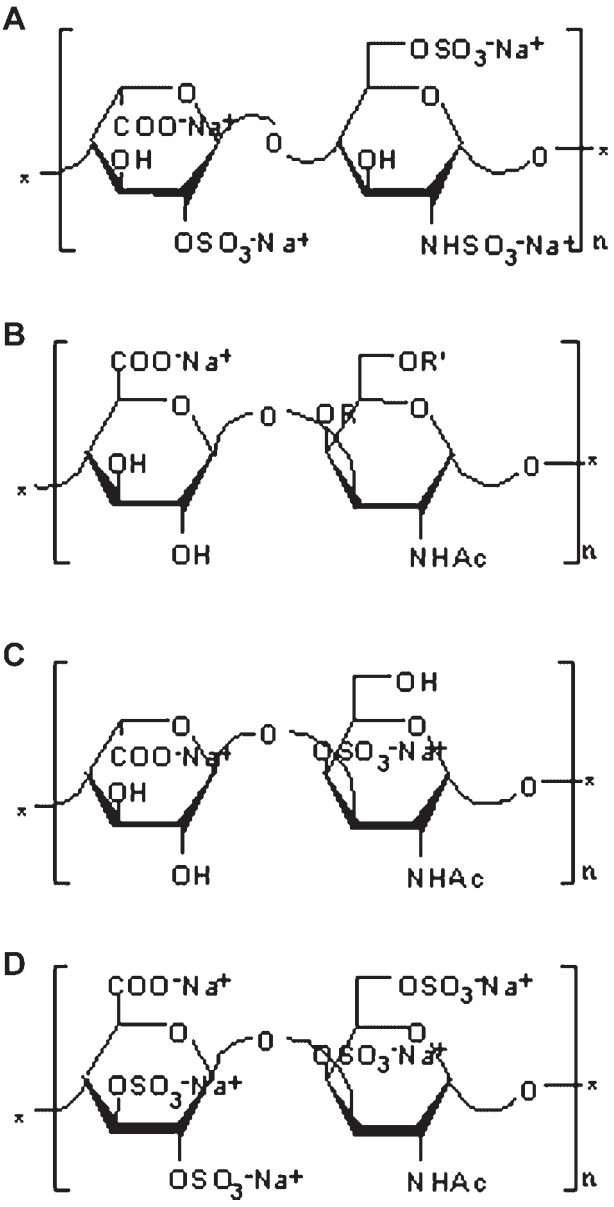

Figure 1. Chemical structures of major repeat units of the sodium salts of (A) heparin, (B) chondroitin sulfate ($R = SO_3^- Na^+$; $R' = H$, CSA; $R = H$; $R' = SO_3^- Na^+$, $^+$, CSC), (C) dermatan sulfate, and (D) oversulfated chondroitin sulfate.

In this article, 31 heparins prepared from 1941 to 2008 and 20 mixtures containing heparin and different heparinoids were analyzed by ^{1}H-NMR. These samples were also treated with nitrous acid and analyzed by polyacrylamide gel electrophoresis (PAGE) to determine the purity of these heparins.

MATERIALS AND METHODS

Materials

Heparin from different sources and years were listed in Table 1. Heparinoids added to heparin as impurities were listed in Table 2.

Methods

NMR

Samples (10 mg) of pure heparin and mixtures (10 mg) of heparin containing 25 wt.% added heparinoid were dissolved in 0.5 mL of D_2O (99.996%; Sigma Co., St. Louis, MO) and freeze-dried three times to remove the exchangeable protons. The samples were redissolved in 0.5 mL of D_2O. Spectra were recorded at 300 K on Bruker Avance II 600, 800 MHz spectrometers equipped with cryogenically cooled HCN probes with *z*-axis gradients. ^{1}H-NMR spectra were recorded on batches of heparins.

Treatment of Heparin With and Without Impurities by Nitrous Acid

OSCS (2, 0.4, and 0.08 mg) is dissolved in 80, 16, and 3 μL of water, respectively. Sulfuric acid (20, 4, and 1 μL of 0.5 M) was added, followed by 1.4, 0.3, and 0.06 mg of sodium nitrite, respectively, to each sample (final molarities were sulfuric acid: 0.1 M, and of the sodium nitrite: 0.2 M). The reactions, performed at room temperature (~22°C), were accompanied by the release of gaseous nitrogen and were stopped 15 min later by

Table 1. Heparins Produced in Different Years

Number	Year	Source	Pure by NMR	Impurity	Comments
1	1941	Bovine lung	Yes		First international std. Hp
2	1959	Bovine lung	Yes		Second international std. Hp
3	1963	Bovine intestinal mucosa	No	HS[a]	First British std. Hp
4	1968	Whale intestinal mucosa	No	HS[a]	MRC, NIMR, UK
5	1983	Porcine intestinal mucosa	Yes		From US market
6	1984	Porcine intestinal mucosa	Yes		From US market
7	1984	Porcine intestinal mucosa	Yes		Fourth international std. Hp
8	1985	Ovine	Yes		From New Zealand
9	1985	Porcine intestinal mucosa	Yes		From US market
10	1986	Porcine intestinal mucosa	Yes		From US market
11	1989	Porcine intestinal mucosa	No	CH_3COONa	From US market
12	1989	Porcine intestinal mucosa	Yes		From US market
13	1996	Porcine intestinal mucosa	Yes		From US market
14	1997	Porcine intestinal mucosa	Yes		Fifth international std. Hp candidate
15	1997	Porcine intestinal mucosa	Yes		Fifth international std. Hp candidate
16	1997	Porcine intestinal mucosa	Yes		Fifth international std. Hp
17	1997	Bovine lung	Yes		Fifth international std. Hp candidate
18	1997	Porcine intestinal mucosa	Yes		Fifth international std. Hp candidate
19	1997	Porcine intestinal mucosa	Yes		Fifth international std. Hp candidate
20	1997	Porcine intestinal mucosa	Yes		Fifth international std. Hp candidate
21	1998	Porcine intestinal mucosa	Yes		From US market
22	1998	Porcine intestinal mucosa	Yes		From US market
23	1999	Porcine intestinal mucosa	Yes		From US market
24	2000	Porcine intestinal mucosa	No	CS	From US market
25	2000	Porcine intestinal mucosa	No	DS	From US market
26	2006	Porcine intestinal mucosa	Yes		From US market
27	2007	Porcine intestinal mucosa	Yes		From US market
28	2008	Porcine intestinal mucosa	No	CH_3COONa	Sixth international std. Hp candidate
29	2008	Porcine intestinal mucosa	Yes		Sixth international std. Hp candidate
30	2008	Porcine intestinal mucosa	Yes		Sixth international std. Hp candidate
31	2008	Porcine intestinal mucosa	No	OSCS	From US market

[a]Signals associated with undersulfation may be attributable to either the presence of HS or unusual heparin structures, such as heparin having reduced 6-sulfation, resulting in some ambiguity of interpretation.

Table 2. Heparinoids as Potential Contaminant in Heparin

Heparinoids	Source	Supplier	MW	Formula	Nitrous Acid Sensitivity[a]	Approx. LOD by NMR of 10 mg Sample[b]	Approx. LOD by PAGE of 100 μg Sample[b]	Approx. Anticoagulant activity (%) of Heparin[c]
Chondroitin sulfate A (CS-A)	Bovine trachea	Celsus	20K	(-4)GlcA(1-3) GalNAc4S(1-)	–	1	1	1
Dermatan sulfate (DS, CS-B)	Porcine intestinal mucosa	Celsus	30K	(-4)IdoA(1-3) GalNAc4S(1-)	–	1	1	1
Chondroitin sulfate C (CS-C)	Shark cartilage	Sigma	20K	(-4)GlcA(1-3) GalNAc6S(1-)	–	1	1	<1
Chondroitin sulfate D (CS-D)	Shark cartilage	Seikagaku	20K	(-4)GlcA2S(1-3) GalNAc6S(1-)	–	1	1	4
Hyaluronic acid (HA)	Rooster comb	Sigma	100K	(-4)GlcA(1-3) GlcNAc(1-)	–	0.5	2	1
Heparan sulfate (HS)	Porcine intestinal mucosa	Celsus	14.8K	(-4)GlcA(IdoA)(1-4) GlcNAc(NS,6S)(1-)	+	10	5	3–15
Heparosan	*E. coli* K5	Our lab	40K	(-4)GlcA(1-4) GlcNAc(1-)	–	10	2	<1
Chitosan sulfate	Semi-synthesis	Our lab	30K	(-4)GlcNS2S3S6S(1-)	++	1	5	12–25
Dermatan disulfate (DS-diS)	Semi-synthesis	Celsus	33K	(-4)IdoA(1-3) GalNAc4S6S(1-)	–	1	0.5	4
Oversulfated HA (OSHA)	Semi-synthesis	Our lab	87K	(-4)GlcA2S3S(1-3) GlcNAc4S6S(1-)	–	0.5	0.5	32–45
Oversulfated DS (OSDS)	Semi-synthesis	Our lab	25K	(-4)IdoA2S3S(1-3) GalNAc4S6S(1-)	–	0.5	0.5	31–40
Oversulfated CS (OSCS)	Semi-synthesis	Our lab	18K	(-4)GlcA2S3S(1-3) GalNAc4S6S(1-)	–	0.5	0.5	45
Dextran sulfate	Semi-synthesis	MP Biomedical	400–600K	(-6)Glc2S3S4S(1-)	–	1	2	7–32
κ-Carrageenan	*Eucheuma cottonii*	Sigma	600–700K	(-3) Gal4S(1-4) 3,6anhydro-Gal(1-)	–	10	2	<1
Fucoidan	*Fucus vesiculosus*	Sigma	40K	(-3)Fucose4S(1-)	–	1	1	20–85
Pentosan sulfate	Semi-synthesis	Sigma	4-6K	(-4)Xyl2S3S(1-)	–	1	1	1–5
PI88	Semi-synthesis	Our lab	2.1 K	(-3)Man 2S4S6S (1-)	–	1	0.1	15–50
Poly(vinyl sulfate)	Synthesis	Sigma	170K	$-CH_2CH(OSO_3)-$	–	0.5	2	>100
Polyanetholesulfonate	Synthesis	Sigma	9-11K	$-CH_2CH(CH_3OC_6H_3(SO_3))-$	–	0.5	2	>100
Sucrose octasulfate (SOS)	Semi-synthesis	Sigma	1.159K	Glc2S3S4S6S(1-2) Fruc1S3S4S6S	–	0.5	0.1	<1

[a]Sensitivity to nitrous acid: –, insensitive; +, some linkages cut; ++, most linkages cut.
[b]The minimum wt.% of a contaminant detectable in a heparin sample.
[c]The percentage of heparin's anticoagulant activity on a wt. basis calculated from reported values[8,30–36] of activated thromboplastin time or plasma-based anti-factor IIa activity assays.

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adjusting the pH to 7–7.2 with 0.1 M of NaOH solution. H_2O was added to the reaction mixtures bunging the volume of each to 0.5 mL. An equal volume of each sample (5 μL) was used ready for PAGE containing 20, 5, and 0.8 μg of sample, respectively.

Mixtures of contaminated heparin (2 mg), containing 25%, 10%, 5%, 1%, and 0% OSCS were treated by nitrous acid (as described above). The products were recovered by the centrifugal filtration (YM-3, 3000 MWCO; Millipore, Bedford, MA), and H_2O was added bunging the reaction mixtures to 0.1 mL. An equal volume of each sample (5 μL) was used for PAGE containing 25, 10, 5, 1, and 0 μg of sample, respectively.

Polyacrylamide Gel Electrophoresis (PAGE)

All batches and impure heparins and degraded samples were analyzed using PAGE (Mini-Gel Apparatus; Bio-Rad, Hercules, CA). An equal volume of each sample (5 μL) was combined with one volume of 50% (w/v) sucrose, and the mixture was loaded into a stacking gel of 5% (total acrylamide) and fractionated on a 15% or 22% resolving gel.[24–26] Electrophoresis was performed at 200 V for 30 min for 15% gel and 80 min for 22% gel. The gel was fixed and stained with Alcian Blue in 2% (v/v) acetic acid. The PAGE was scanned, digitized, and analyzed by the software UN-scan-it gel (Silk Scientific, Orem, UT). All experiments are performed in triplicate.

RESULTS AND DISCUSSION

Heparin contaminated by OSCS killed nearly 100 Americans in early 2008. This impurity was detected and its structure was elucidated by NMR. The ^{1}H-NMR spectra of pure heparin and OSCS-contaminated heparin are shown in Figure 2. The ^{1}H-NMR of heparin (Fig. 2A) shows that it consists of a major IdoA2S-GlcNS6S repeating disaccharide unit and minor amounts of GlcA, IdoA, GlcNAc, and GlcNS residues. Contaminated heparin gave a ^{1}H-NMR showing nine additional peaks (Fig. 2B). A detailed assignment of this contaminant which has been previously published[7,8,23] confirmed that these nine peaks belonged to OSCS. Multiple lots of heparin were similarly analyzed by ^{1}H-NMR and most of these samples showed only small difference in their saccharide composition, corresponding to a variation in peak area (Tab. 1 and Fig. 3).

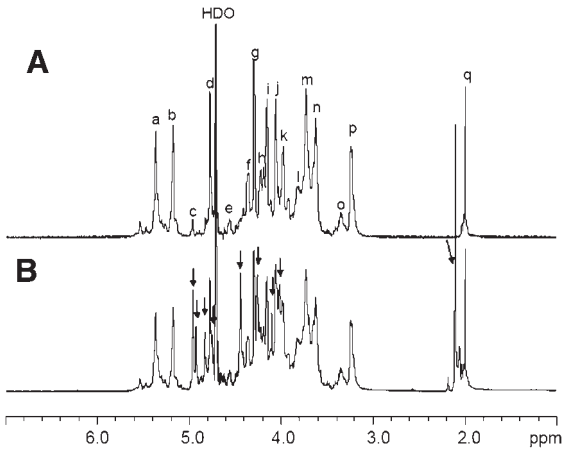

Figure 2. ^{1}H-NMR spectra (800 MHz) of (A) heparin and (B) OSCS-contaminated heparin. a, H1 GlcNS, GlcNS6S; b, H1 IdoA2S; c, H1 IdoA; d, H5 IdoA2S; e, H1 GlcA; f, H6 GlcNS6S; g, H2 IdoA2S; h, H6′ GlcNS6S; i, H3 IdoA2S; j, H4 IdoA2S; k, H5 GlcNS5S; l, H6 GlcNS; m, H4 GlcNS6S; n, H3 GlcNS, GlcNS6S; o, H2 GlcA; p, H2 GlcNS6S; q, methyl signals of acetyl group. Arrows indicate peaks associated with OSCS contaminant.

Based on the ^{1}H-NMR spectra of 31 heparins, the chemical shifts for peaks a–q in heparin were always present at 5.42, 5.23, 5.01, 4.82, 4.60, 4.40, 4.34, 4.27, 4.12, 4.03, 3.87, 3.77, 3.67, 3.40, 3.28, and 2.05 ppm. While the relative intensities of these 16 peaks varied, the chemical shifts differ by $<$0.03 ppm. Three areas in the ^{1}H-NMR spectra of heparin samples, from 0.10 to 2.00, 2.10 to 3.20, 5.60 to 8.00 ppm, are relatively free of peaks. Thus, any signals observed in these areas likely correspond to impurities or contaminants. The heparin samples containing impurities or contaminants are shown in Figure 4. The spectra of heparins 3 and 4, prepared in 1964 and 1968, show two additional peaks at 3.8 and 5.3 ppm assigned as the peak of H1 and H6 of a glucosamine lacking sulfo groups at the 6-position indicative of the presence of heparan sulfate (HS)[27] or simply an undersulfated heparin with a reduced number of 6-*O*-sulfo groups. The distinction here is difficult to make as HS is naturally found in tissues containing heparin and has a lower charge density than heparin. While HS may be present in all heparins, heparins 3 and 4 are either particularly rich in HS or are heparins having a reduced sulfation. An extra peak observed at 1.9 ppm in the spectra of heparins 11 and 28 corresponds to the presence of a sodium acetate impurity. Heparins 24 and 25 contain more than 15% CS and DS, respectively. A peak corresponding to the

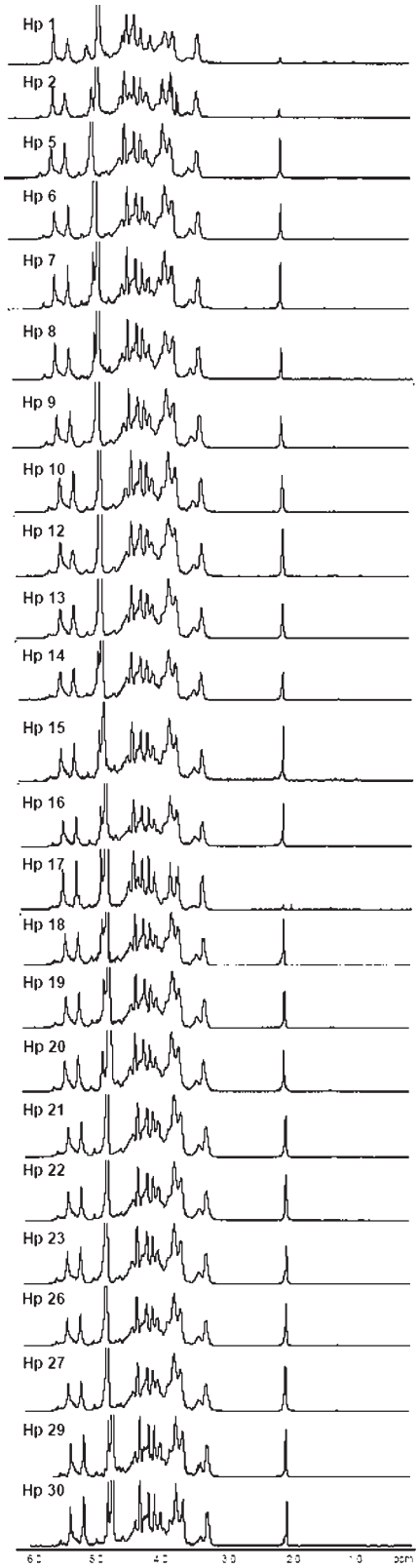

Figure 3. ^{1}H-NMR spectra (600 MHz) of 30 pure heparin samples.

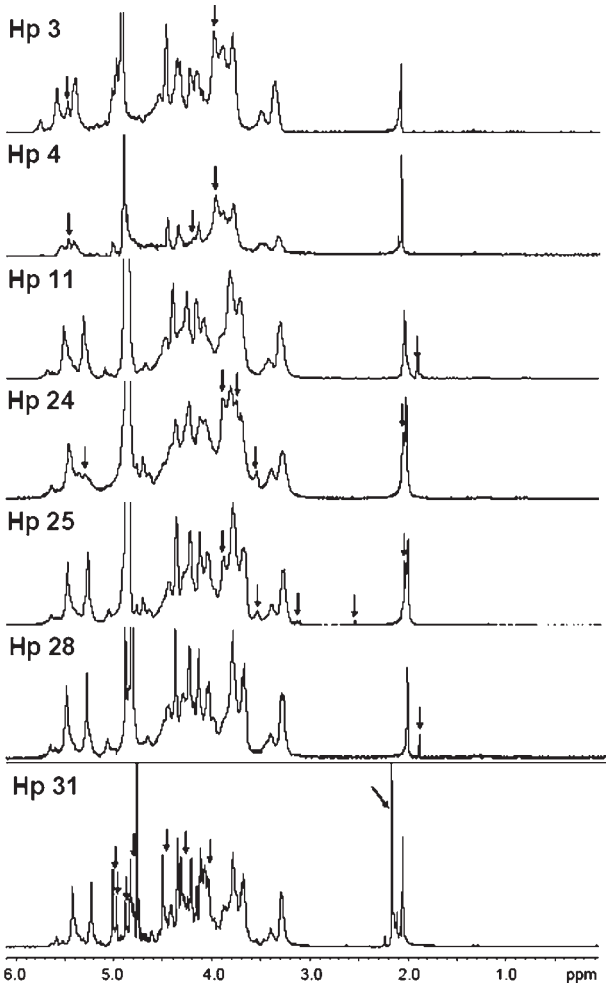

Figure 4. ^{1}H-NMR spectra (600 and 800 MHz) of seven heparin samples showing unusual structural features, impurities, and/or contaminants. Arrows indicate suspect peaks.

methyl signal of the CS/DS is clearly observed at 2.08 ppm. The peaks at 3.55 and 3.60 ppm are assigned as H-2 of GlcA and IdoA of CS impurity in heparin 24 and DS impurity in heparin 25, respectively. Perlin and coworkers[9] first reported the NMR detection of DS impurities in heparin. Heparin 31 contains OSCS based on the presence of a peak at 2.15 ppm. The full-scale NMR spectra are shown in Figure 2S in Supplementary Data.

In addition to OSCS, HS, CS, and DS, many additional natural and artificial heparinoids, some with anticoagulant activities similar to heparin, have been prepared or synthesized. A group of these heparinoids were added to heparin (heparin containing 25 wt.% heparinoid) and analyzed by ^{1}H-NMR (Fig. 5). The methyl signal of the acetyl peaks of CS-A, -B, -C, and -D are

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observed at ~2.08 ppm, at a slightly lower field than observed for those of heparin at 2.05 ppm. In contrast, the methyl signal of the acetyl group of HA is observed at higher field strength, 1.94 ppm. The chemical shifts of the methyl signals of acetyl groups of oversulfated CS/DS/HA are observed at lower field strengths, 2.15 ppm for OSCS, 2.14 ppm for OSDS, and 2.17 ppm for OSHA. The ^{1}H-NMR spectra of HS and heparosan are similar and overlap with many of the peaks observed in the spectrum of heparin. In contrast to heparin, however, HS and heparosan's peaks corresponding to positions with lower sulfation have a greater peak area. The anomeric proton of chitosan sulfate, dextran sulfate, and pentosan sulfate resonate down field from the anomeric proton of heparin. Higher field peaks are also observed in poly(vinyl sulfate) and polyanethole sulfate, and lower field peaks are observed for the phenyl groups of polyanethole sulfate (the full-scale NMR spectra are shown in Fig. 3S in Supplementary Data). Carrageenan and fucoidan, having higher molecular weights, show broader peaks and considerable overlap with heparin. Higher field signals corresponding to methyl groups of fucose residues are also observed in fucoidan. PI-88 and SOS are oligosaccharides, of low molecular weight and, thus, show sharper peaks in their ^{1}H-NMR spectra. Thus, all of the heparinoids examined show additional peaks, which are clearly distinguishable from heparin. Many of these additional peaks resonate at field strengths in the three peak-free areas (0.10–2.00, 2.10–3.20, and 5.60–8.00) of the ^{1}H-NMR spectrum of heparin. Some of them were observed close to the peaks of heparin. The limits of detection (LOD) limit for these impurities as determined in 10 mg samples by 600 MHz ^{1}H-NMR varied between 0.5% and 10%. This variation arises from the presence of significant contaminant peaks, such as acetyl methyl signals or an anomeric proton signals, in one of the three peak-free areas of the heparin spectrum. The LOD for each contaminant is listed in Table 2. The ^{1}H-NMR spectra shown in Figure 5 (also see Fig. 3S in the Supplementary Data) serve as reference spectra. They are useful for identifying the heparinoids when they contaminated heparin. Such reference spectra should be helpful in monitoring the quality of heparin API.

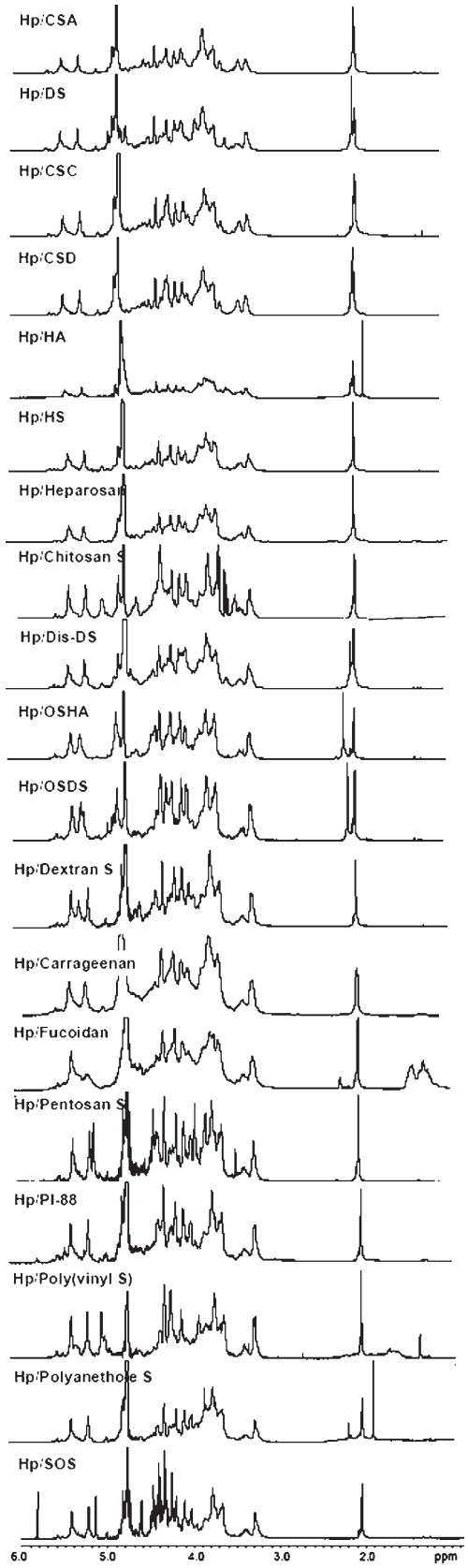

Figure 5. ^{1}H-NMR spectra (600 MHz) of pure heparin to which various heparinoids were added at 25 wt.%.

While ^{1}H-NMR can be used to detect and quantify many contaminants in heparin, this method is dependent on signals from the contaminant falling into areas of the heparin spectrum that are relatively free of peaks. Moreover, ^{1}H-NMR requires milligram amounts of heparin and relatively specialized equipment, a high-field NMR spectrometer. Thus, we decided to also examine the utility of PAGE analysis, a routine and inexpensive method of analysis available in most biochemical laboratories for the semi-quantitative determination of contaminants in heparin. PAGE analysis can be made even more selective by analyzing both untreated (intact) heparin sample and nitrous acid-treated samples.

OSCS in heparin could be detected by PAGE after nitrous acid treatment.[23] Nitrous acid depolymerization is relatively specific for heparin, due to the presence of nitrous acid-sensitive *N*-sulfo groups in heparin, that are absent on OSCS and most heparinoids, which contain hexosamine residues substituted by *N*-acetyl groups.[28,29] Using nitrous acid followed by PAGE analysis, OSCS was quantified in different proportions in mixtures with heparin. Semi-quantitative gel analysis can also be performed as the standard curves show excellent linearity (see Fig. 1S in Supplementary Data).

Nitrous acid also detects 17 heparinoids (Tab. 2 and Fig. 6). It is no surprise that HS and chitosan sulfate, having *N*-sulfo groups, are partially depolymerized by nitrous acid treatment. Only a very lightly stained smear at the top of the gel was observed for both samples after nitrous acid treatment. HS, containing *N*-sulfo domain and *N*-acetyl domains, was partially degraded. The resistant *N*-acetyl domains, having less sulfo groups, could not be easily visualized by Alcain blue staining. Chitosan sulfate, having 80% *N*-sulfo domain, was nearly entirely degraded,

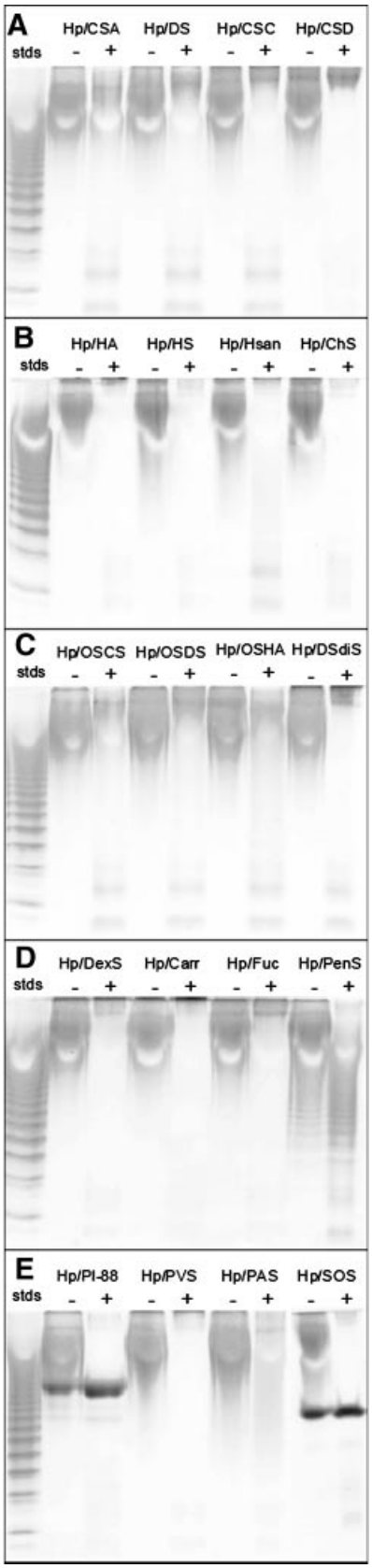

Figure 6. PAGE analysis of mixture of heparinoids and heparin after nitrous acid treatment. The first lane in each gel (A–E) contains mixture of heparin oligosaccharide standards (degree of polymerization (dp) 4 (the band running the furthest into the gel) through 18 corresponding to tetrasaccharide through octadecasaccharide). The remaining lanes in each gel contain mixtures of heparin and heparinoid at (25 wt.%) untreated (−) and nitrous acid treated (+). Hsan, heparosan; Chs, chitosan sulfate; Dexs, dextran sulfate; Carr, carrageenan; Fuc, fucoidan; PenS, pentosan sulfate; PVS, poly(vinyl sulfate); PAS, polyanetholesulfonate.

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with only 20% resistant *N*-acetyl domain remaining at the top of the gel. The remaining heparinoids contain only *N*-acetyl groups or have no amino sugars and, thus, are stable to nitrous acid treatment and are easily detected on PAGE after heparin is degraded by nitrous acid. The LOD of these impurities (Tab. 2), using PAGE followed by Alcain blue staining, depends on their molecular weight, negative charge density (staining intensity) and separation from heparin or nitrous acid depolymerized heparin.

Many of the impurities examined reportedly have anticoagulant activity as measured by either activated partial thromboplastin time or by plasma-based anti-factor IIa assays.[8,30–36] These activities range from <1% of heparin's anticoagulant activity to >100% of that activity. With the exception of HS, which has a specific pentasaccharide sequence (also present in heparin) that facilitates antithrombin-mediated inhibition of factors IIa and Xa, the reported activities of the remaining potential contaminants appear to act primarily through heparin cofactor II-mediated inhibition of factor IIa. While activity measurements can assist in identifying impure or contaminated heparins, care must be taken to select the appropriate assays. Global assays, such as the USP clotting assay in place during the 2008 contamination crisis, are not ideally suited for the detection of contaminants and impurities exhibiting anticoagulant activity.

In conclusion, vigilance is still required to keep the supply of heparin safe and effective. Multiple orthogonal assays including spectroscopy, separation-based methods, and activity assays offer an excellent approach.

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