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ORIGINAL PAPER

Structural characterization of heparins from different commercial sources

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Abstract Seven commercial heparin active pharmaceutical ingredients and one commercial low molecular weight from different manufacturers were characterized with a view profiling their physicochemical properties. All heparins had similar molecular weight properties as determined by polyacrylamide gel electrophoresis (M_N, 10–11 kDa; M_W, 13–14 kDa; polydispersity (PD), 1.3–1.4) and by size exclusion chromatography (M_N, 14–16 kDa; M_W, 21–25 kDa; PD, 1.4–1.6). one-dimensional ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) evaluation of the heparin samples was performed, and peaks were fully assigned using two-dimensional NMR. The percentage of glucosamine residues with 3-*O*-sulfo groups and the percentage of *N*-sulfo groups and *N*-acetyl groups ranged from 5.8–7.9%, 78–82%, to 13–14%, respectively. There was substantial variability observed in the disaccharide composition, as determined by high performance liquid chromatography (HPLC)-mass spectral analysis of heparin lyase I–III digested heparins. Heparin oligosaccharide mapping was performed using HPLC following separate treatments with heparin lyase I, II, and III. These maps were useful in qualitatively and quantitatively identifying structural differences between these heparins. The binding affinities of these heparins to antithrombin III and thrombin were evaluated by using a surface plasmon resonance competitive binding assay. This study provides the physicochemical and activity characterization necessary for the appropriate design and synthesis of a generic bioengineered heparin.

Keywords Heparin · Polyacrylamide gel electrophoresis · Size exclusion chromatography analysis · Molecular weight properties · Disaccharide composition · High performance liquid chromatography–mass spectrometry · Oligosaccharide mapping · Nuclear magnetic resonance spectroscopy · Surface plasmon resonance

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Introduction

Heparin and heparin-derived low molecular weight heparins (LMWHs) are the most widely used clinical anticoagulants [1]. Modern medical procedures including treatment of thrombotic disorders, such as deep vein thrombosis, extracorporeal therapy, such as renal dialysis and blood oxygenation, the use of indwelling catheters and shunts, and the post-surgical control of clots have increased the need for heparin and LMWH [2, 3]. The introduction of modern

medical procedures in developing countries has further increased the demand for heparin. Heparin is a highly sulfated, linear glycosaminoglycan that is abundantly found in mucosal tissues such as the lungs and intestines [4]. The structure of this ~10–20 kDa polysaccharide is predominantly made up of a major repeating disaccharide unit, α-L-IdoA2S (1→4)-α-D-GlcNS6S (where IdoA is idopyranosyluronic acid, S is sulfo, and GlcN is 2-deoxy, 2-amino glucopyranose) [1]. The structure and sulfation pattern of the heparin molecule are integral to its therapeutic value [5]. In particular, a unique pentasaccharide sequence present in heparin having the structure →GlcNAc6S→GlcA→GlcNS3S6S→IdoA2S→GlcNS6S→ (where GlcA is D-glucopyranosyluronic acid and Ac is acetyl) is responsible for its specific binding to the serine protease inhibitor antithrombin III (AT) resulting in its conformational activation and leading to the inhibition of major coagulation cascade proteases, including thrombin (factor (F) IIa) and FXa [1]. Heparin lyases I, II, and III are important tool for heparin structural analysis. The activity of heparin lyases relies on the cleavage of specific glycosidic linkages at C4 of uronic acid residues present in heparin through an eliminase mechanism, resulting in unsaturated oligosaccharide products that have UV absorbance spectra maxima at 232 nm.

Heparin is currently produced from the tissues of food animals, primarily pig intestine, sheep intestine, and beef lung. There are drug safety concerns related to heparin products. For example, the appearance of bovine spongiform encephalopathy in Europe in the 1990s and the concerns of prion contamination have decreased the production and use of bovine and ovine heparin worldwide [6]. As a result, there has been an increased demand on porcine intestinal heparin with over half the current world supply coming from China. The appearance of blue-ear disease, a viral infection that killed up to 10% of the pigs in China [7], further stressed the tight supply of animal-sourced heparin. A lack of control on Chinese heparin production resulted in the introduction of oversulfated chondroitin sulfate (OSCS) contaminated product in 2007–2008, associated with numerous adverse reactions and multiple deaths [8, 9]. This comes at a time of critical need for a reliable and controlled source of heparin that is uniform in structure and activity and free of viral, prion, and chemical contaminants.

An improved understanding of heparin biosynthesis [10, 11], the availability of all the heparin biosynthetic enzymes as recombinant proteins expressed in *Escherichia coli* [12, 13], and a method for cofactor recycling [14] has led to the preparation of multi-milligram quantities of heparin that is chemically similar to animal-sourced USP heparins in our laboratory [15, 16]. Our ultimate goal is to prepare a bioengineered heparin that is chemically, biologically, and pharmacologically identical to animal-sourced heparin. The introduction of a generic bioengineered heparin requires the use of sophisticated chemical analysis as well as in vitro and in vivo (animal and human) studies. Because heparin is a polydisperse mixture of polysaccharide chains with sequence heterogeneity, a multifaceted approach is required for their chemical and structural analysis. This paper examines seven heparins in the form of active pharmaceutical ingredient (API) collected from a variety of manufacturers and one LMWH for comparison. The physicochemical properties including molecular weight, disaccharide composition, oligosaccharide maps, chemical fine structure, and thrombin and AT binding affinity were characterized to provide the basis for the design and preparation of a generic bioengineered heparin.

Experimental procedures

Materials

Seven heparin sodium API (Table 1, no. 1–3, 5–8) and one LMWH API (no. 4) produced by nitrous acid depolymerization were provided by manufacturers from USA, China, and Europe. Sodium heparin used in the protein binding studies (200 IU/mg) was from Celsus (Cincinnati, OH, USA). Recombinant flavobacterial heparin lyase I, II, and III were expressed in our laboratory using *E. coli* strains, provided by Professor Jian Liu (University of North Carolina, College of Pharmacy, Chapel Hill, NC, USA) [12]. Reagents for polyacrylamide gel electrophoresis, Alcian blue dye, 2-cyanoacetamide, tetra-*n*-butylammonium hydrogen sulfate, and other reagents used in this study were from Sigma (St. Louis, MO, USA). Heparin oligosaccharides, from hexasaccharide to icosasaccharide, were used as calibrants for molecular weight determination by size exclusion chromatography (SEC) and were purchased from Iduron (Manchester, UK). Unsaturated hepa-

Table 1 Anti-factor IIa and anti-factor Xa activities provided by the manufacturers

Heparin no.	Anti-factor IIa activity (IU/mg)[a]	Anti-factor Xa activity (U/mg)	Anti-Xa/ Anti-IIa ratio[b]	Source
1	197	197	1.0	USA
2	203	–	–	Ireland
3	201	197	0.98	USA
5	203	–	–	Spain
6	204	–	–	Spain
7	210	–	–	Spain
8	200	–	–	China

[a] USP heparin is required to have an anti-factor IIa activity of >180 IU/mg

[b] USP heparin is required to have an anti-factor Xa/anti-factor IIa activity ratio of 0.9 to 1.1

rin/HS disaccharides standards (Di-0S, ΔUA-GlcNAc; Di-NS, ΔUA-GlcNS; Di-6S, ΔUA-GlcNAc6S; Di-UA2S, ΔUA2S-GlcNAc; Di-UA2SNS, ΔUA2S-GlcNS; Di-NS6S, ΔUA-GlcNS6S; Di-UA2S6S, ΔUA2S-GlcNAc6S; and Di-triS, ΔUA2S-GlcNS6S, where ΔUA is deoxy-α-L-threo-hex-4-enopyranosyl uronic acid) were obtained from Seikagaku Corporation (Japan).

Polyacrylamide gel electrophoresis (PAGE) analysis Heparin samples were separated by PAGE on a 12% total acrylamide (12% T) resolving gel containing 11.8% (*w*/*v*) acrylamide, 0.74% (*w*/*v*) *N*,*N*′-methylene-bis-acrylamide, and 5% (*w*/*v*) sucrose. All monomer solutions were prepared in resolving buffer (0.1 M boric acid, 0.1 Tris, 0.01 M disodium EDTA, pH 8.3). Stacking gel monomer solution was prepared in resolving buffer with the pH adjusted to 6 using HCl, and it contained 4.75% (*w*/*v*) acrylamide and 0.25% (*w*/*v*) *N*,*N*′-methylenebisacrylamide. Gels were subjected to electrophoresis at a constant power of 200 V for 25 min, stained by Alcian blue for 30 min, and destained in water. PAGE images were acquired on an HP Scanjet 7400 at 600 dpi and processed for densitometry data using Un-Scan-it software (Silk Science Inc., Orem, UT, USA). These data were used to calculate weight averaged molecular weight (M_W), number averaged molecular weight (M_N), and polydispersity (PD) of pharmaceutical heparin [17].

Size exclusion chromatography (SEC) of heparin SEC was performed using a TSK-GEL G3000PWxl size exclusion column with a sample injection volume of 20 μl and a flow rate of 0.6 ml/min on an apparatus composed of a Shimadzu LC-10Ai pump, a Shimadzu CBM-20A controller, and a Shimadzu RID-10A refractive index detector [18]. The mobile phase consisted of 0.1 M $NaNO_3$. The column was maintained at 40 °C with an Eppendorf column heater during the chromatography. The SEC chromatograms were recorded with the LCsolution Version 1.25 software and analyzed with its "GPC Postrun" function. Heparin oligosaccharides of different molecular weights (degree of polymerization (dp)6, dp10, dp16, and dp20) were used as calibrants for the standard curve.

Disaccharide analysis using LC/MS The heparin lyase I, II, and III (10 mU each) in 5 μl of 25 mM Tris, 500 mM NaCl, and 300 mM inidazole buffer (pH 7.4) were added to 5 μg heparin samples in 25 μl of distilled water and incubated at 37 °C for 10 h. The products were recovered by centrifugal filtration (YM-10), and the HS/heparin disaccharides were recovered in the flow-through, freeze-dried for LC-MS analysis.

LC-MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE, USA) equipped with 6300 ion trap, binary pump followed by a high-pressure UV detector [19]. The column used was an Acquity UPLC BEH C18 column (2.1×150 mm, 1.7 μm, Waters, Milford, MA, USA). Eluent A was water/acetonitrile (85:15) *v*/*v*, and eluent B was water/acetonitrile (35:65) *v*/*v*. Both eluents contained 12 mM TrBA and 38 mM NH_4OAc with pH adjusted to 6.5 with HOAc. A gradient of solution A for 10 min and followed by a linear gradient from 10 to 40 min was used at flow rate of 100 μl/min for disaccharide analysis. The column effluent entered the source of the ESI-MS for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source of temperature of 350 °C, to obtain the maximum abundance of the ions in a full scan spectrum (200–1,500 Da). Nitrogen was used as a drying (8 l/min) and nebulizing gas (40 psi).

Quantification analysis of heparin/HS disaccharides was performed by using calibration curves constructed by separation of increasing amounts of unsaturated heparin/HS disaccharide standards (2, 5, 10, 15, 20, 30, 50, and 100 ng/per each disaccharide). The linear equation was based on amount of disaccharide and peak intensity in mass spectrometry. All analyses were performed in triplicate.

Heparin oligosaccharide mapping using analytical strong anion exchange (SAX)-HPLC Each individual heparin sample (4.0 mg) was dissolved in 4.0 mL of 50 mM, pH 7.5, sodium phosphate buffer and was incubated in a 30 °C water bath with heparin lyase 1 (Hep I, 1.5 unit, activity against heparin), heparin lyase 2 (Hep II, 0.35 unit, activity against heparin), or heparin lyase 3 (Hep III, 5.0 unit activity against heparan sulfate) [20]. The reaction completion was moni-

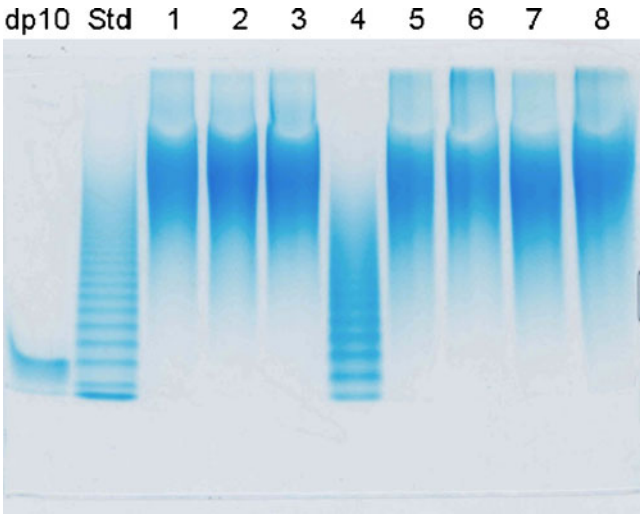

Fig. 1 Polyacrylamide gel electrophoresis (PAGE) analysis on heparin samples. *Lanes* are *dp10*, corresponding to the decasaccharide $\Delta UA2S(GlcNS6S\text{-}IdoA2S)_4GlcNS6S$; *Std*, an oligosaccharide ladder prepared by partial (30%) Hep I depolymerization of bovine lung heparin; *lanes 1* to *8*, correspond to heparin samples no. 1 to 8 (sample no. 4 is LMWH)

Table 2 PAGE analysis of heparin samples for weight averaged molecular weight (M_W), number averaged molecular weight (M_N), and polydispersity (PD)

Heparin sample no.	M_W[a]	M_N	PD
1	14,000±1,000	10,200±2,300	1.41
2	13,700±800	10,400±2,000	1.35
3	13,700±900	10,200±2,300	1.37
4	6,300±400	4,600±200	1.38
5	13,700±800	10,100±1,200	1.36
6	15,700±1,400	11,200±2,100	1.42
7	13,400±1,000	10,100±1,600	1.34
8	14,600±1,100	10,800±1,500	1.37

[a] Standard deviation based on triplicate measurements

Table 3 SEC of commercial heparins for molecular weight determination (average error based on duplicate measurements)

Heparin sample no.	M_W	M_N	PD
1	22,900±100	15,400±190	1.49
2	20,900±600	15,000±500	1.39
3	21,900±300	15,900±100	1.38
4	6,400±200	4,700±100	1.38
5	21,300±300	14,300±80	1.49
6	24,400±1,100	15,500±200	1.57
7	21,000±20	14,600±100	1.44
8	24,000±400	14,600±300	1.64

tored by taking out small amount of aliquots of the reaction mixture for PAGE analysis. The same amount of each heparin lyase was added to the reaction at each 12-h interval for another two times for an exhaustive digestion on the substrates. Reactions were quenched by heating in a 100 °C water bath for 10 min.

SAX-high performance liquid chromatography (HPLC) analysis of heparin lyase digestion products were performed on a Shimadzu LC-10Ai LC system equipped with an SPD-20A ultraviolet–visible (UV) detector using a 4.6×250-mm Waters Spherisorb S5 SAX column. A two-segment gradient elution was achieved using mobile phase A (water, pH 3.5, adjusted with HCl) and mobile phase B (2.0 M NaCl, pH 3.5, adjusted with HCl) at a flow rate of 1.0 ml/min. Ten microliters of each disaccharide standard was loaded onto the column in a concentration of 0.5 μg/μl and washed with 0% to 60% B over 60 min. The elution was monitored at 232 nm.

Nuclear magnetic resonance (NMR) analysis Approximately 20 mg of each porcine mucosal heparin samples from different companies were analyzed by one-dimensional (1D) ^{1}H, ^{13}C, and two-dimensional (2D) correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), nuclear Overhauser effect spectroscopy (NOESY), and total correlation spectroscopy (TOCSY) experiments [21]. All samples were dissolved in 0.5 ml of 99.996% deuterium oxide ($^{2}H_2O$, Sigma, St. Louis, MO, USA) and freeze dried to remove exchangeable protons. NMR experiments were obtained at a Bruker Avance Ultrashield 600 MHz (14.1-Tesla) NMR instrument equipped with an ultrasensitive HCN cryoprobe with a *z*-axis gradient. The spectra were acquired at a probe temperature of 298 K. Proton and carbon resonances were assigned with the use of

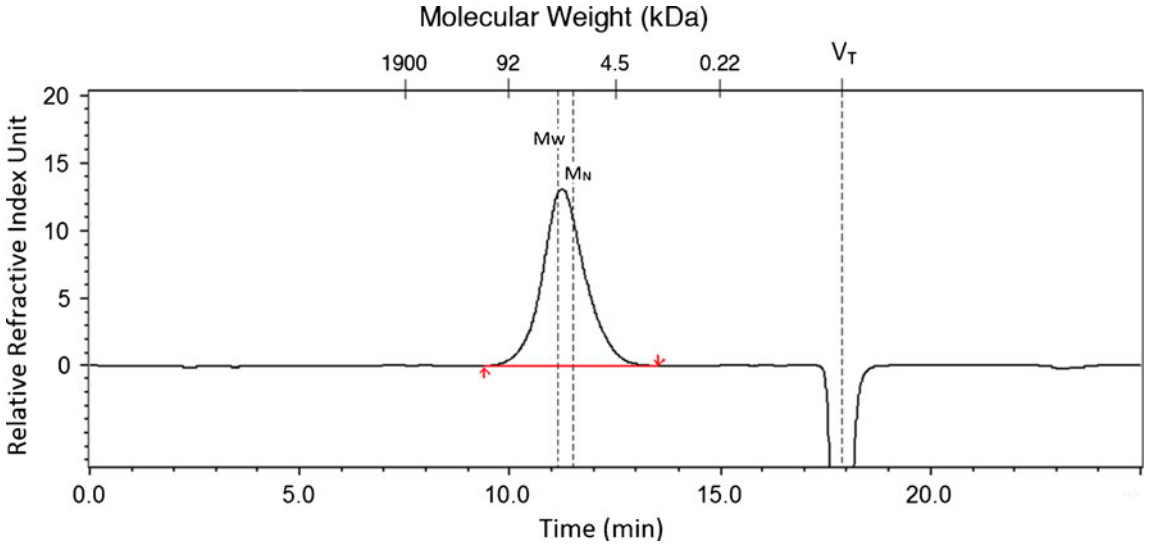

Fig. 2 A sample chromatogram using SEC for heparin molecular weight determination of heparin no. 1. The *y*-axis corresponds to the mass detected using a refractive index detector, and the *x*-axes correspond to elution time (minutes) and molecular mass (calculated from a standard curve). V_T marks the total volume of the column where a change in salt concentration results in a negative peak

conventional HMQC, COSY, and TOCSY spectra. For 1D ^{1}H-NMR spectra, sweep width of 20.5 ppm and acquisition time of 2.65 s were used. Sweep width was 236 ppm, and number of scans was 20,480 for 1D ^{13}C spectra. For the COSY, TOCSY, and NOESY spectra, 512 experiments resulting in 4,096 data points for a spectral width of 12.4 ppm were measured. Proton-detected HMQC experiments used 12.4 and 78.8 ppm spectral widths in the ^{1}H dimension and ^{13}C dimension, respectively. A mixing time of 250 ms with 1.5 s relaxation delay was used in NOESY experiment. The 2D NMR data sets were processed by Topspin version 2.1.4.

Surface plasmon resonance (SPR) analysis Biotinylated heparin prepared from Celsus heparin was immobilized to sensor chip SA (streptavidin; GE Healthcare, Uppsala, Sweden) based on the manufacturer's protocol. SPR measurements were performed on a BIAcore 3000 (GE Healthcare, Uppsala, Sweden) operated using the version software. Solution competition study between surface heparin and soluble different heparins to measure IC_{50} were performed using SPR [22]. AT (250 nM) mixed with different concentrations (range from 0, 1.25, 2.5, to 5.0 U/ml) of heparin in HBS-EP buffer (10 mM 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), 150 mM sodium chloride, 3 mM ethyl-

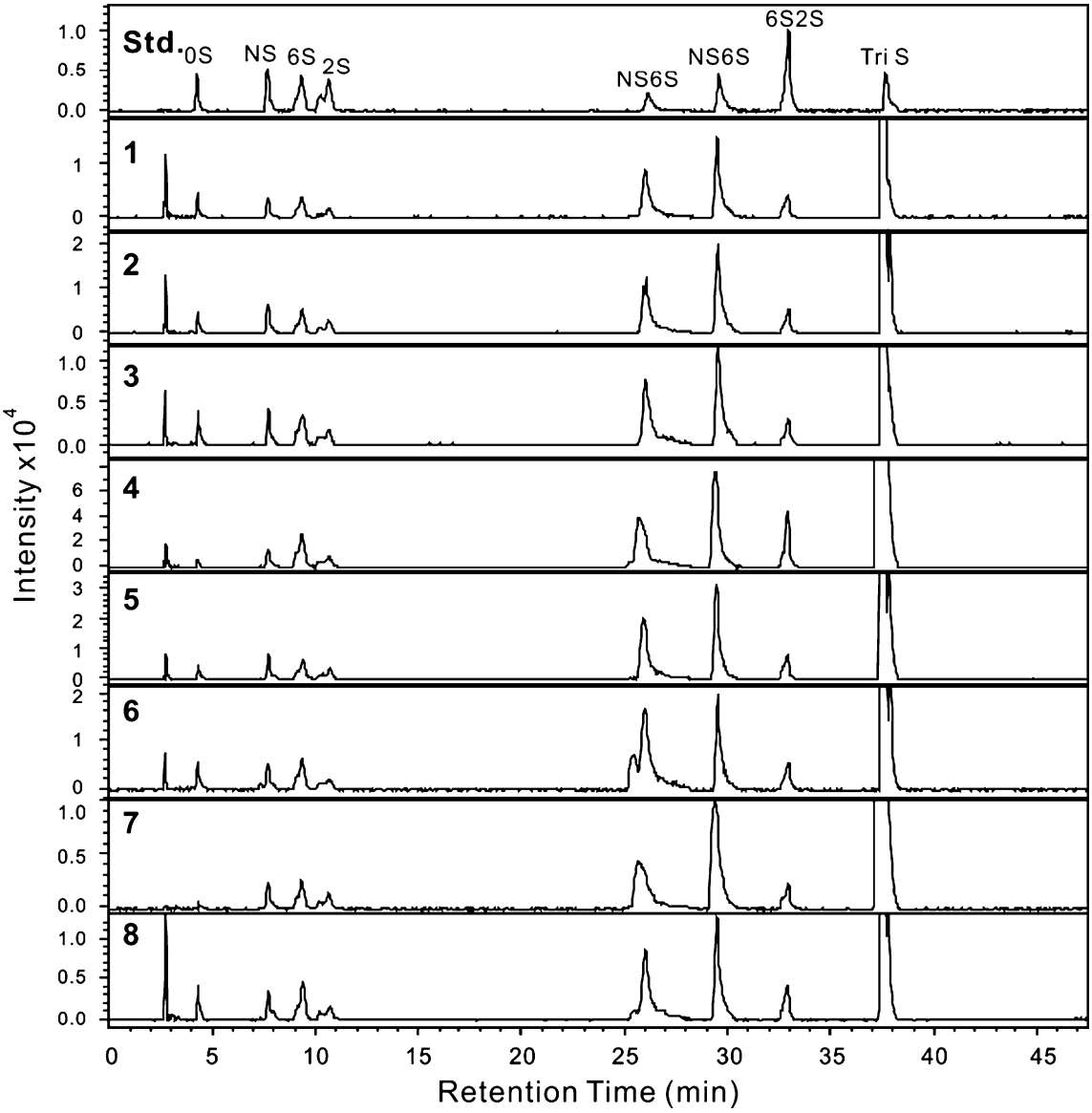

Fig. 3 EIC of disaccharide analysis of seven heparin samples and one LMWH sample by RPIP-UPLC-MS. *Std* shows the analysis of an equimolar mixture of eight disaccharide standards used for peak identification and injection of different amounts afford a standard curve for quantification. Chromatograms *1–8* show the disaccharide analysis of the eight commercial heparin samples no. 1–8, respectively. The peak eluting prior to 0S in each chromatogram is salt. The small peak eluting just prior to NS6S in heparin no. 1 and no. 8 was tentatively identified as ΔUA2SGlcNH$_2$6S based on its mass

enediaminetetraacetic acid (EDTA), 0.005% polysorbate surfactant P20, pH 7.4; GE Healthcare, Uppsala, Sweden) were injected over heparin chip at a flow rate of 30 μl/min, respectively. After each run, the dissociation and the regeneration were performed as described above. For each set of competition experiments on SPR, a control experiment (only protein without heparin) was performed to make sure the surface was completely regenerated and that the results obtained between runs were comparable. The same analysis was performed with 63 nM thrombin in place of the AT.

Results and discussion

Selection of heparin samples for study Eight heparin API samples were obtained in 2009–2010 from different international manufacturers. These heparins complied with the pharmacopeial standards in effect in the countries in which they are sold. Seven of the eight heparins (no. 1–3 and 5–8) had anti-factor IIa activities, provided by the manufacturers of >180 IU/mg consistent with the current USP requirements for anti-factor IIa activity.

Molecular weight properties of heparin samples PAGE was used to determine the number averaged molecular weight (M_N) and weight averaged molecular weight (M_W), as well as the polydispersity (PD), of the heparin samples [17]. Furthermore, PAGE offers a qualitative map of the components within heparin and can be used to establish the presence of polyanionic impurities including OSCS [23]. The analysis of these heparins all show a broad, continuous, and virtually identical smears of unresolved polysaccharide bands indicating the high and uniform level of microheterogeneity of these preparations (Fig. 1). Qualitative evaluation of Fig. 1 confirms that 1–3 and 5–8 are heparin, and that 4 is a LMWH, and that none of the samples show the presence of obvious impurities or contaminants. Molecular weight is calculated based on a banding ladder of heparin oligosaccharides (Std in Fig. 1) prepared through the partial heparin lyase I depolymerization of heparin [17]. The size of the oligosaccharides present in this banding ladder begins by identifying one of the bands by using a pure heparin decasaccharide (labeled dp 10 in Fig. 1). Alignment of the dp standard affords a counting frame from which the bands in the Std lane of Fig. 1 can be identified and assigned a molecular mass required for the preparation of a standard curve. The LMWH, shown in lane 6, is clearly distinguished from heparin by both its lower molecular weight and the characteristic banding pattern associated with the presence of chains having only an even number of saccharide units (heparin shows no clear banding due to the presence of even and odd saccharide chains) [17]. PAGE analysis indicated that all seven heparin samples had M_N of 10–11 kDa, M_W of 13–14 kDa, and PD of 1.3–1.4 (Table 2).

Next, the molecular weight properties of the heparin samples were assessed using SEC, this time using individual heparin oligosaccharide standards. A typical SEC chromatogram for heparin no. 1 is shown in Fig. 2. SEC analysis showed that all heparins had M_N of 14–16 kDa, M_W of 21–25 kDa, and PD of 1.4–1.6 (Table 3). The molecular weight properties determined by PAGE and SEC (Tables 2 and 3) while similar are not identical, with SEC showing slightly higher molecular weights and polydispersities. The differences are method associated as PAGE assumes equal staining intensity of different chain sizes [17], and SEC can be complicated by interactions between the heparin polyelectrolyte and the supporting column matrix [24]. Currently, it is not possible to obtain mass spectral data on intact heparin chains capable of affording a standard-free characterization of heparin's molecular weight. Despite these complicating issues, both PAGE and SEC values are consistent with those reported in the literature (Table 1) [17, 25].

Table 4 Disaccharide compositional analysis of seven heparin samples and one LMWH sample

Heparin sample no.	Disaccharide composition (%)[a]							
	ΔDi-0S	ΔDi-NS	ΔDi-6S	ΔDi-2S	ΔDi-NS6S	ΔDi-NS2S	ΔDi-6S2S	ΔDi-TriS
1	3.2 (1.0)	2.9 (0.35)	2.3 (0.29)	1.0 (0.1)	6.7 (0.51)	3.7 (0.30)	0.77 (0.06)	79.4 (1.81)
2	1.1	1.8	2.1	1.6	8.5	9.2	2.1	73.6
3	1.8	2.2	3.2	1.6	10.1	11.6	2.6	66.9
4	0.3	0.4	1.6	0.7	4.9	5.1	2.1	84.9
5	0.6	1.1	1.6	1.1	6.7	7.4	1.8	79.7
6	1.1	1.6	2.3	1.0	10.8	8.9	1.9	72.4
7	0.3	0.8	1.2	0.8	5.4	6.6	1.1	83.8
8	1.4	1.4	2.6	1.1	8.8	7.8	2.5	74.4

[a] Analyses were performed in singlicate except for heparin no. 1, which was performed in triplicate to assess the variability of disaccharide analysis. Standard deviations for each disaccharide from sample no. 1 are shown in parentheses

Disaccharides analysis Heparin is composed of different disaccharide units [1]. The most prominent of these is the trisulfated disaccharide α-L-IdoA2S (1→4)-α-D-GlcNS6S. On treatment with a mixture of heparin lyase I, II, and III (Hep I, II, and III), heparin is broken into unsaturated disaccharides [26] (a small quantity of resistant tetrasaccharide associated with the AT binding site is also formed) [27]. The major trisulfated disaccharide affords the ΔUA2S (1→4)-α-D-GlcNS6S disaccharide (where ΔUA corresponds to 4-deoxy-α-L-*threo*-hex-4-eno-pyranosyluronic acid). After treatment with heparin lyases, the heparin and LMWH samples were analyzed reversed phase ion pairing (RPIP)-ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS). The extracted ion chromatograms (EIC) of disaccharides analyses are presented in Fig. 3. Eight common heparin disaccharides were detected in all samples; their quantity was calculated from a linear equation derived from a calibration curve of these eight disaccharide standards [28], and their disaccharide compositions are given in Table 4. Analyses show that the major disaccharide in heparin is the trisulfated disaccharide as expected, but it is present in a range from 63 to 85 mol%, suggesting that manufacturing processes and/or source material have an impact disaccharide composition. The highest value of

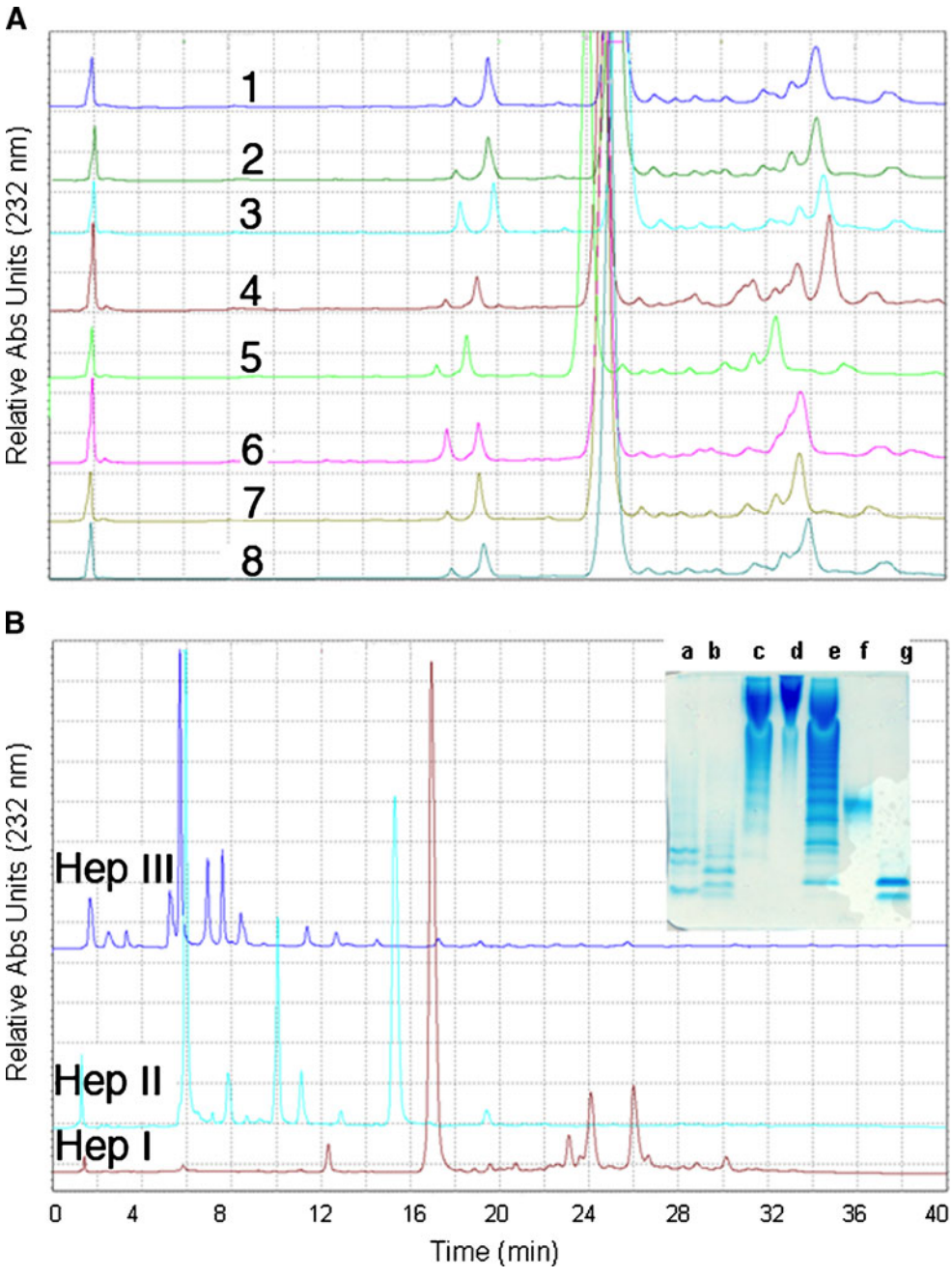

Fig. 4 Quantitative disaccharide mapping of heparins using SAX-HPLC. (**A**) SAX-HPLC analysis of heparins no. 1–3 and 5–8 and LMWH no. 4 digested by Hep I. (**B**) HPLC analysis of heparin no. 1 digested by HepI, HepII, and HepIII, respectively. *Insert*: PAGE analysis of digested heparin; *lanes* (**a**–**c**), heparin no. 1 digested by Hep I, II, and III, respectively; (**d**) heparin no. 1; (**e**) oligosaccharide ladder (see Fig. 1 legend); (**f**) oligosaccharide standard dp10 (see Fig. 1 legend); (**g**) oligosaccharide standard dp4 (most intense band corresponds to ΔUA2SGlcNS6S-IdoA2SGlcNS6S)

Table 5 SAX-HPLC analysis of seven commercial heparin samples digested with Hep I, II, or III

	Heparin sample no.							Average (%)	Std. dev.
	1 (%)	2 (%)	3 (%)	5 (%)	6 (%)	7 (%)	8 (%)		
Heparin lyase I									
2a[c]	0.87[a]	0.86	0.75	0.78	0.77	0.81	0.79	0.80	0.05[b]
2b	1.22[a]	1.01	1.18	0.99	1.05	1.08	1.15	1.10	0.09[b]
2c	4.44[a]	4.65	4.51	4.55	3.99	4.11	3.98	4.32	0.30[b]
2d	80.40[a]	79.7	79.08	80.07	80.89	80.58	80.85	80.22	0.70[b]
Heparin lyase II									
2a	0.93	0.89	0.97	0.98	0.95	1.10	1.02	0.98	0.07
2b	15.5	14.50	15.30	14.90	15.20	15.10	13.50	14.86	0.70
2c	9.10	9.00	10.10	8.90	8.10	7.90	7.40	8.64	0.90
2d	61.60	62.02	60.54	63.24	62.29	64.13	65.97	62.83	1.8
2e	3.51	3.31	3.49	3.41	3.11	2.99	2.89	3.24	0.30
2f	3.11	2.99	3.08	3.02	3.01	2.70	3.09	3.00	0.10
2g	1.44	1.69	1.72	1.71	1.70	2.10	1.79	1.74	0.20
2h	1.41	1.40	1.45	1.39	1.42	1.51	1.40	1.43	0.04
Heparin lyase III									
2a	43.19	42.69	43.46	49.78	43.50	52.05	39.76	44.92	4.3
2b	4.66	4.28	3.85	4.06	5.70	4.30	4.75	4.51	0.60
2e	12.76	12.73	11.99	11.60	12.25	11.77	14.25	12.48	0.90
2f	11.61	11.2	12.1	13.92	12.75	13.91	15.20	12.96	1.5

[a] Peak area integrations of major oligosaccharide compositions were quantified and sum to 100%. Quantitative analysis of the oligosaccharide products was performed in triplicate, and the std. dev. ranged from 0.02% to 9.7% of the reported values

[b] Analysis was performed on each chromatogram, and the average value of each oligosaccharide standard in the seven heparins treated with Hep I, II, or III was calculated, and the standard deviations across the seven heparin samples were calculated

[c] The structures determined for each peak are 2a, ΔUA-GlcNAc; 2b, ΔUA-GlcNS6S; 2c, ΔUA2S-GlcNS; 2d, ΔUA2S-GlcNS6S; 2e, ΔUA-GlcNS; 2f, ΔUAGlcNAc6S; 2g, ΔUA2S-GlcNAc; 2h, ΔUA2S-GlcNAc6S

84.9 mol% trisulfated disaccharide was obtained for heparin 4, which had been shown by PAGE to be a LMW heparin. This increased level of trisulfated sequence could result either from some selectivity in the chemistry used in the depolymerization reaction of result from a recovery or purification step that enriches highly sulfated chains.

Heparin oligosaccharide mapping Heparin samples were next individually digested by each of the three heparin lyases (Hep I, II, and III) and were analyzed in triplicate by SAX-HPLC to obtain quantitative oligosaccharide maps (Fig. 4). This mapping technique is similar to that used to prepare peptide maps of proteins and is useful for comparing structural differences among heparins [29]. The assigned disaccharide peaks, where pure standards were available, were integrated with their mole percentages with respect to the total disaccharides present in each mixture determined (Table 5). Each heparin lyase affords a unique map containing different disaccharides (of known structures) and oligosaccharides (some of known structure) [30], providing more structural information for each individual heparin sample than would a simple disaccharide analysis [20]. Heparin lyase I selectively cleaves heparin's most highly sulfated sequences releasing four disaccharides, most prominently trisulfated disaccharide 2d, while heparin lyase III is selective for its low sulfate domains, affording four disaccharides, most prominently unsulfated disaccharide 2a. Heparin lyase II shows the lowest selectivity affording a complex mixture of eight different disaccharides 2a–2h (Fig. 4 and Table 5). The relative amount of certain disaccharides varied significantly between the different commercial heparin samples providing a qualitative evaluation of heparin variability based on the manufacturing process and animal/tissue source of each heparin (for example, some porcine heparins come from whole intestine, while others are derived only from porcine intestinal mucosa [31]). The manufacturing process may also modify the structure of the core protein linkage region in the reducing end of intact heparin, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser (where GlcA is D-glucuronic acid, Gal is D-galactose, Xyl is D-xylose, and Ser is serine) affording modified tetrasaccharide fragments of the linkage region

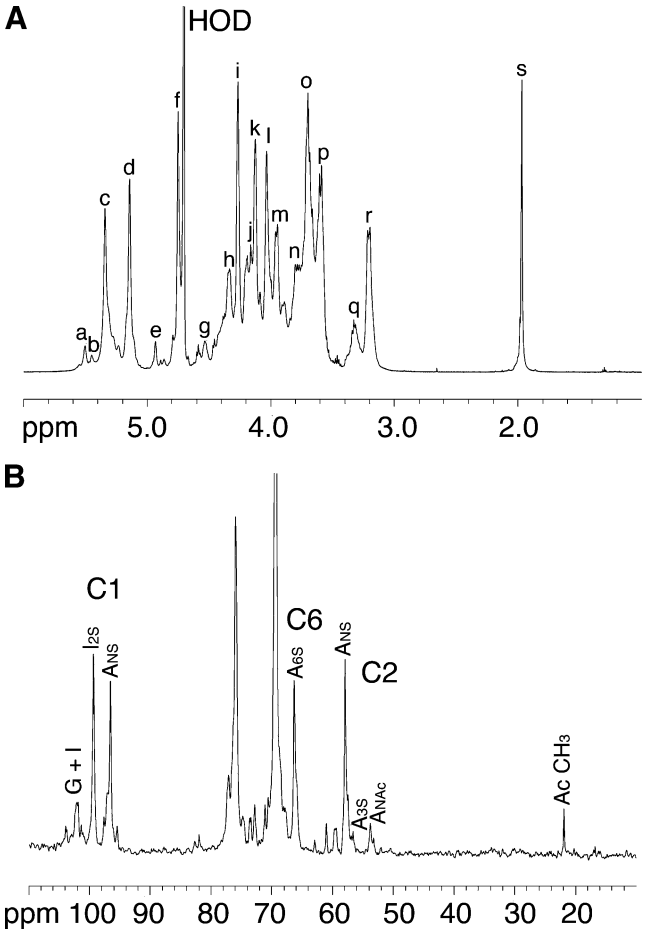

Fig. 5 A sample NMR analysis of a pharmaceutical heparin ^{1}H-NMR and ^{13}C-NMR spectra of heparin no. 1. The spectra were acquired at 600 MHz on a Bruker Avance Ultrashield 600 MHz (14.1-Tesla) NMR instrument. (**A**) Peak assignments for proton spectrum: (**a**) H1 A_{NS}-(G); (**b**) H1 A_{3S}; (**c**) H1 A_{NS6x}; (**d**) H1 I_{2S}; (**e**) H1 I; (**f**) H5 I_{2S}; (**g**) H1 G; (**h**) H6 A_{NS6S}; (**i**) H2 I_{2S}; (**j**) H6′ A_{NS6S}; (**k**) H3 I_{2S}; (**l**) H4 I_{2S}; (**m**) H5 A_{NS6S}; (**n**) H6 A_{NS}; (**o**) H4 A_{NS6S}; (**p**) H3 A_{NS6x}; (**q**) H2 G, H2 A_{3S}; (**r**) H2 A_{NS6x}; (**s**) acetyl CH_3 (*A*, glucosamine; *I*, iduronic acid; *G*, glucuronic acid). (**B**) ^{13}C-spectrum of heparin no. 1 (20 mg) in 0.5 mL of deuterium oxide

such as ΔUA-Gal-Gal-Xyl-*O*-$CH_2CONHCH_2COOH$. The amount of linkage region fragments in the seven heparin samples ranged from ~1% to ~1.3%.

Fine structural analysis 1D ^{1}H and ^{13}C NMR evaluation of the heparin samples was performed with and without prior lyophilization to allow the detection of volatile solvents such as ethanol (Fig. 5a, b). Process impurities were present in all seven heparin samples. Half of these contained ethanol, while the other half contained sodium acetate. All peaks were fully assigned (Table 6) by 2D NMR including HMQC (Fig. 6), HHCOSY, TOCSY, and NOESY studies. Critical features in the GlcN residues, including *N*-sulfo, *N*-acetyl, and 3-*O*-sulfo, 6-*O*-sulfo content vary amongst heparins. Critical features in the uronic acid residues including the content of IdoA, IdoS2S, and GlcA also show a range of values (Table 6).

Surface plasmon resonance (SPR) analysis Two proteins (AT and thrombin), having important roles in coagulation system, were used for the heparin binding SPR experiments. Solution/surface competition experiments were performed by SPR to examine the relative binding affinity of different heparins to AT and thrombin interaction. AT (250 nM) mixed with different concentrations of heparin in HBS-EP buffer were injected over heparin chip. Once the active binding sites on AT molecules were occupied by heparin in the solution, the binding of AT to the surface-immobilized heparin should decrease resulting in a reduction in SPR. The IC_{50} values (concentration of competing analyte resulting in a 50% decrease in response units (RU)) can be calculated from the plots (AT or human thrombin binding signal (normalized) versus heparin concentration in solution). The injections were performed in duplicate (with less than 5% variation). Similar solution/surface competition experiments were conducted with human thrombin. The calculated IC_{50} values for different heparins are shown in Table 7. While these binding data show a weak correlation to in vitro anti-factor Xa and

Table 6 Percent substitution of glucosamine (A) and uronic acids (iduronic acid I or glucuronic acid G) in heparin samples no. 1–8

Heparin sample no.	A_{NS}%	A_{NAc}%	A_{3S}%	A_{6S}[a]%	I_{2S}%	I%	G%	Impurities observed
1	81	13	6.2	83.4	61.3	11.5	27.2	Ethanol
2	80	13	7.9	83.4	60.8	10.0	29.1	Acetate
3	78	14	7.8	82.0	61.8	9.5	28.6	Ethanol
4[b]	80	14	6.6	87.2	57.0	13.5	29.5	Ethanol
5	80	14	6.0	83.7	62.6	10.3	27.0	Acetate
6	81	13	6.3	84.1	54.2	11.9	33.9	Acetate
7	82	13	5.8	82.9	65.3	9.9	24.8	Acetate
8	80	13	6.9	84.1	60.9	10.7	28.4	Ethanol
Avg.	80	13	6.7	83.4	61	10.5	28.4	
Std. dev.	0.9	0.5	0.7	0.6	2.6	0.7	2.0	
Var.	0.7	0.2	0.4	0.3	5.8	0.4	3.4	

[a]Measurements were made in singlicate, but integration values are generally accurate within 5%. A_{6S} values obtained by the integration of the ^{13}C spectra

[b]Sample no. 4 is LMWH

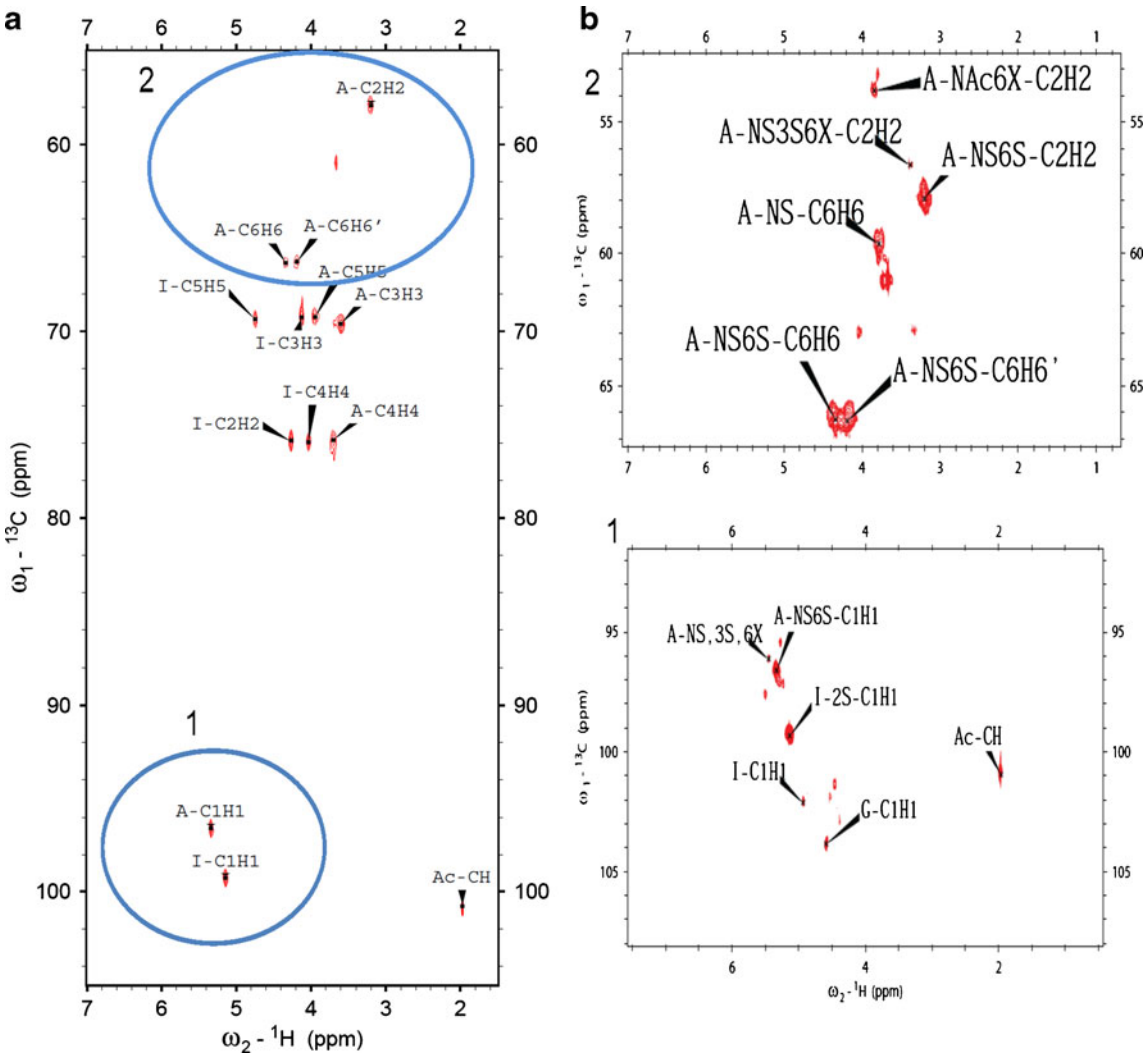

Fig. 6 Two-dimensional ^{1}H-^{13}C correlation spectrum (HMQC) of heparin no. 1. (**a**) Entire HMQC spectrum. (**b**) The selected regions of the spectrum. *Blue circles 1* and *2* indicate anomeric region and H2/C2-H6/C6 glucosamine resonances, respectively

anti-factor IIa activities [22], their relationship to heparin's in vivo activity, if any, is currently unclear.

Conclusions

The contemporaneous (prepared after the end of the heparin crisis of 2008) heparin APIs examined were prepared from animals raised on three continents and on heparins processed in different manufacturing facilities using different processes. Despite these differences in source material and process, there was a surprising similarity in the physicochemical characteristics of these products. There were, however, unique features associated with each heparin API that begins to define the acceptable range of structural features that define pharmaceutical heparins that have qualified as EP and USP heparin. This information

Table 7 IC_{50} of different commercial heparins measured by solution/surface competition SPR

Heparin sample no.	IC_{50} (U/ml) to AT[a]	IC_{50} (U/ml) to human thrombin[a]
1	1.8	1.5
2	1.3	1.8
3	1.2	1.3
5	1.2	1.5
6	1.5	2.1
7	1.1	1.8
8	0.9	1.7

[a] These are relative values for binding to AT and thrombin in competition with a single immobilized heparin. These values are based on duplicate experiments with less than 5% of errors

should help inform our evaluation of the chemical equivalence of generic heparin preparations, particularly the bioengineered heparin currently being developed in our laboratory [32]. Studies are currently underway to evaluate the in vitro and in vivo activities and the pharmacodynamics and pharmacokinetics of these heparin samples.

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