Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Kishimoto TK, Viswanathan K, Ganguly T, et al. Contaminated heparin associated with adverse clinical events and activation of the contact system. N Engl J Med 2008;358:2457-67. DOI:10.1056/NEJMoa0803200.

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Supplemental Information

- *Sample preparation.* Approximately 15 mg of each UFH sample was dissolved in 0.7 mL of D_2O (99.9%) and lyophilized to dryness. The dissolution and lyophilization process was performed 1-3 times per sample. The dried, exchanged samples were dissolved in 0.7 mL of deuterium oxide (99.96 atom %) and transferred into a 5 mm NMR tube. Before spectral acquisition, each sample was sonicated for approximately 60s to remove air bubbles.
- *1D ^1H NMR analysis.* All spectra were obtained at 303K with presaturation of the residual water signal. Each spectrum was the average of 32 scans, with a 90° excitation angle, 16 ppm sweep width, 1.7 s acquisition time, and 14 s recycle delay. All spectra were processed in MestReC with 0.3 Hz exponential line broadening and baseline correction. The acetyl methyl signal of N-acetyl-glucosamine was referenced to 2.02 ppm.
- *Determination of longitudinal relaxation times*. To ensure accurate identification and quantification, longitudinal relaxation times were determined with an inversion-recovery experiment. All spectra were obtained at 303K with presaturation of the residual water signal. Each spectrum was the average of 4 scans, with a 90° excitation angle, 16 ppm sweep width, 1.7 s acquisition time, and 20 s recycle delay. The acetyl methyl signal of N-acetyl-glucosamine was referenced to 2.02 ppm. The determination of T_1 relaxation times was performed using VnmrJ. All signals of interest were determined to have T_1 values no greater than 2.6 seconds.
- *Titration of contaminants.* 15 mg/ml stock solutions of dermatan sulfate and over-sulfated chondroitin were prepared by dissolution in 99.9% D_2O, lyophilization, weighing, and dissolution in the appropriate amount of 99.96% D_2O. UFH samples were prepared with 15 mg UFH lot 1035-0765 in 0.7 ml D_2O. The impurity stock solutions were gradually added to the UFH samples. 1D NMR spectra were obtained for each of the impurity concentrations. Limit of detection for both dermatan sulfate and OSCS were determined to be ~0.3 wt%.
- *Quantification of Contaminants/Impurities.* **Figure S1** shows a representative one dimensional NMR spectrum. The acetyl signal associated with OSCS is shown as P3; the acetyl signal for dermatan sulfate is shown as P4. Three hexosamine signals are associated with heparin, since it can either be N-acetylated or N-sulfated. Since all species consist of a disaccharide repeat of uronic acid linked a hexosamine, quantification can be completed by using the measured hexosamine concentration. Thus, the following equations were used

The molar concentration of heparin can be determined as follows-

$$C_{UFH} = P1 + P2 + (P5 - P4)/3$$

The molar concentration of dermatan sulfate is -

$$C_{DES} = P4/3$$

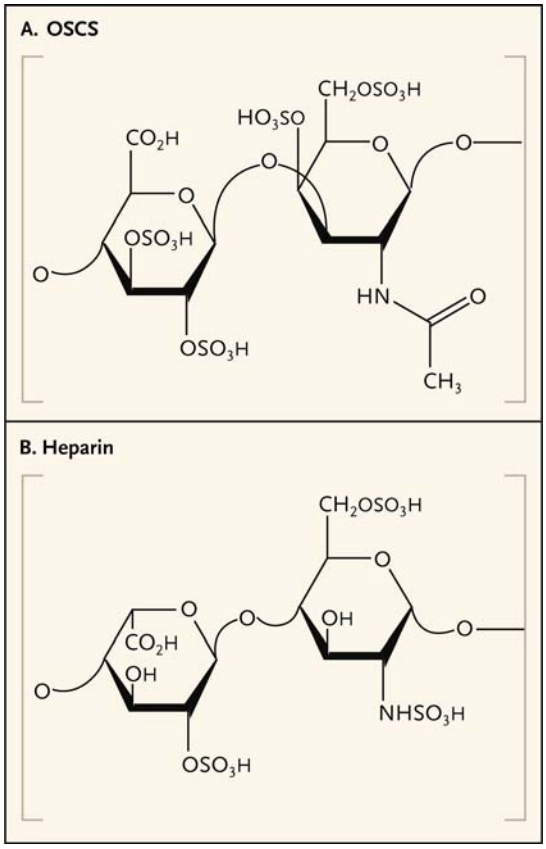
The molar concentration of OSCS is -

$$C_{OSCS} = P3/3$$

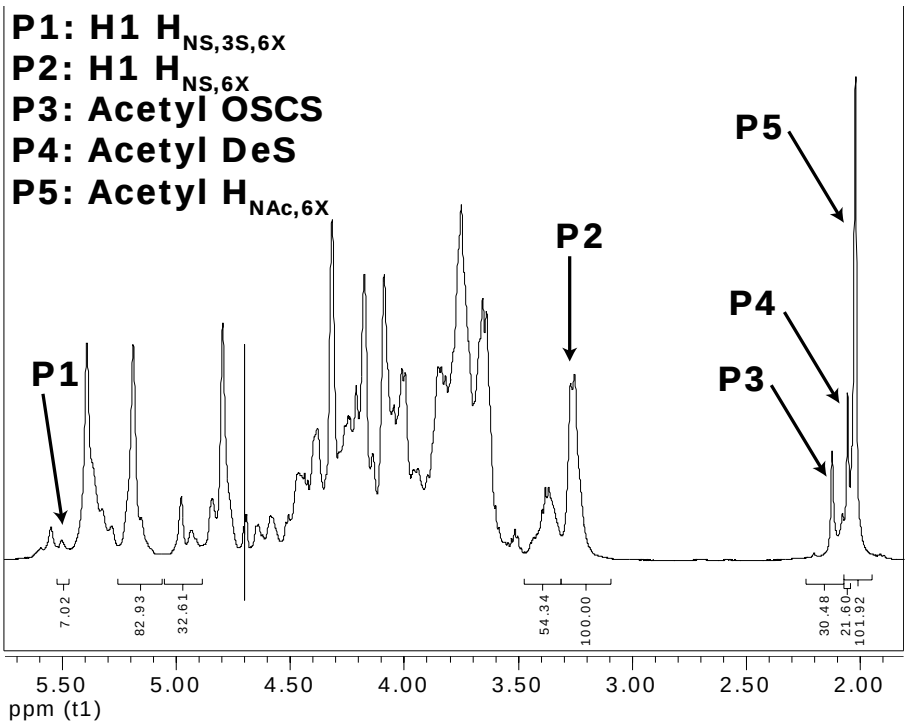
Finally a weight percentage for OSCS can be calculated as follows-

$$wt\%_{OSCS} = \left(C_{OSCS} * DSM_{OSCS}\right) / \left(C_{UFH} * DSM_{UFH} + C_{DeS} * DSM_{DeS} + C_{OSCS} * DSM_{OSCS}\right)$$

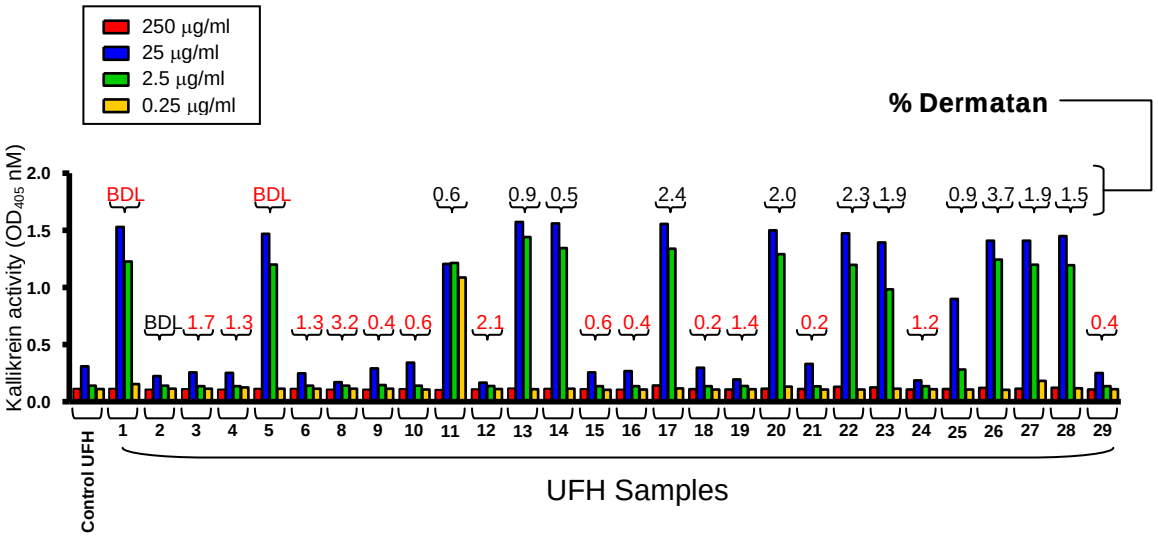
Where DSM is the average disaccharide molar mass of heparin, dermatan sulfate, or OSCS, respectively. The results were corrected for alterations in the baseline levels.



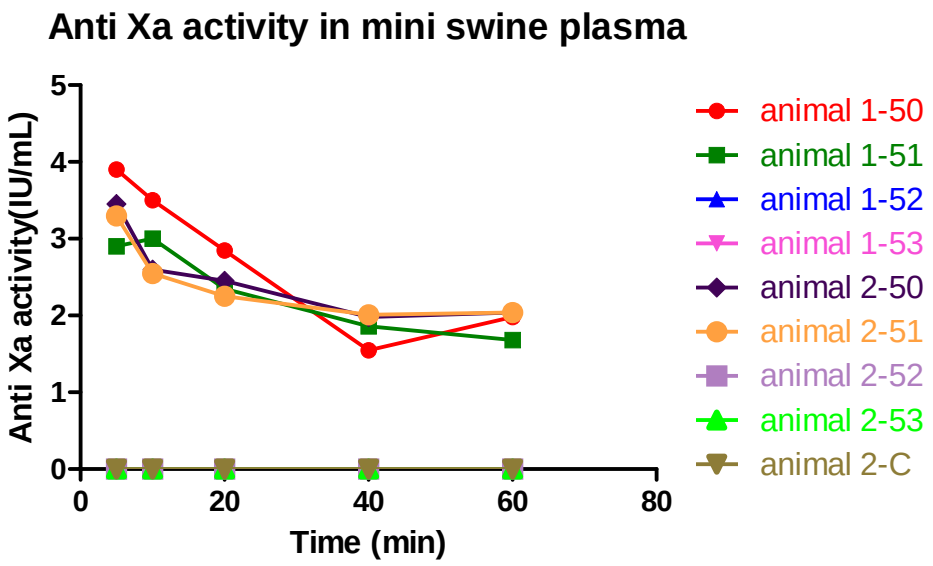
Supplemental Figure 1. *Structures of the disaccharide repeat units for glycosaminoglycans.* (A) The tetrasulfated disaccharide unit repeat for OSCS. (B) The major repeat unit of heparin consists of a trisulfated disaccharide.



Supplemental Figure 2. Representative 1D NMR spectrum of a heparin sample contaminated with OSCS with dermatan sulfate as an impurity.



Supplemental Figure 3. The presence of dermatan sulfate in heparin is not associated with the ability to induce kallikrein activity. Samples of heparin, representing both suspect heparin lots and control lots, were analyzed in a blinded fashion for both the presence of dermatan sulfate and for the ability to activate kallikrein. The presence of dermatan sulfate was detected and quantified by 1D NMR. Kallikrein amidolytic activity was assessed at various concentrations of heparin, as indicated. Below Detection Levels (BDL)



Supplemental Figure 4. Anti-factor Xa activity in pig plasma samples. Pig plasma samples from Figure 5B were analyzed for anti-Xa activity with the Coatest Heparin kit (Chromogenix) on a Coag-A-Mate MTX II instrument. A standard curve based on the activity of enoxaparin was used to interpolate the anti-Xa activity (IU/ml) of the unknown samples.

Supplemental Table 1. *Quantification Results for Sample 1-29 (nd=not detected)*

Sample	OSCS	Dermatan Sulfate
1	19.3%	nd
2	nd	nd
3	nd	1.7%
4	nd	1.3%
5	6.6%	nd
6	nd	1.3%
7	nd	1.3%
8	nd	3.2%
9	nd	0.4%
10	nd	0.6%
11	27.4%	0.6%
12	nd	2.1%
13	12.3%	0.9%
14	14.5%	0.5%
15	nd	0.6%
16	nd	0.4%
17	6.0%	2.4%
18	nd	0.2%
19	nd	1.4%
20	13.7%	2.0%
21	nd	0.2%
22	5.3%	2.3%
23	4.1%	1.9%
24	nd	1.2%
25	2.4%	0.9%
26	6.6%	3.7%
27	18.3%	1.9%
28	11.0%	1.5%
29	nd	0.4%

Analysis of the Heparin Samples. Samples 1-29 were analyzed for the presence of dermatan sulfate and OSCS. The results are shown in **Table 1**; quantification was determined by using the process and equation outlined above.