ABSTRACT

An unexpectedly high rate of adverse events and deaths were reported from November 2007 to April 2008 in patients exposed to heparin. Investigations on the quality of heparin revealed an oversulfated chondroitin sulfate (OCS) contaminant in the heparin. Several reports on the chemical structure and limited biologic actions of OCS have recently become available. However, no data are available on its anticoagulant and antiprotease effects. Moreover, its interaction with heparin, resulting in the modulation of heparin’s anticoagulant activity, is not reported. The isolated contaminant from recalled batches of heparin and several semisynthetic OCSs were profiled for their in vitro anticoagulant effects. The contaminant and OCSs both exhibited measurable anticoagulant activity and produced supra-additive effects in the presence of heparin. In addition, in animal models of thrombosis and bleeding, the contaminated heparin produced stronger anticoagulant and bleeding effects than heparin. Thus, in addition to the reported biological actions, the heparin contaminant exerts measurable anticoagulant effects and interacts with heparin to produce supra-additive effects. These studies suggest that the contamination of heparin with OCSs was based on a rational design and prior knowledge of molecular and anticoagulant profiles. These studies also indicate that the OCS contaminant may itself be heterogeneous and its molecular and biological profiles may vary in different recalled heparins.

KEYWORDS: Oversulfated chondroitin sulfate, contaminant, recalled heparins

Heparin represents a complex mixture of sulfated mucopolysaccharides with an apparent molecular weight range of 14 to 16 kd. Commercially available heparin is obtained either from porcine mucosal or bovine lung tissues. Heparin is a heterogeneous drug composed of several components. The biological actions of heparin are usually measured by using pharmacopeial assays such as the United States Pharmacopeia (USP) sheep plasma assay. Until recently, there was no requirement to provide any structural information on heparin components using such methods as nuclear magnetic resonance (NMR). The quality of heparin varies from...
different suppliers and origins. The USP and EP (European Pharmacopeia) methods primarily require only basic anticoagulant specifications.

Heparin is a widely used anticoagulant drug used for the management of thrombotic and cardiovascular indications. It is also the sole anticoagulant for surgical and procedural uses. Allergic reactions and severe adverse effects such as heparin-induced thrombocytopenia (HIT) and bleeding complications are associated with its use and managed by following certain guidelines. Despite the known adverse reactions, this drug has been beneficial in the anticoagulant management of both medical and surgical patients.

From November 2007 to April 2008, an unexpected increase in adverse events and deaths led to concerns and investigations on the quality of heparin.1-3 Alarmed by public concern, the U.S. Food and Drug Administration recalled certain heparin batches, and along with heparin manufacturers and academia, the composition of the heparins was investigated. Preliminary studies on the recalled heparin lots used in hemodialysis revealed the presence of a high molecular weight contaminant in heparin that was not digestible by heparinase-1. Additional studies on the digestion of this substance with chondroitinases suggested that this may be a modified form of chondroitin sulfate such as its oversulfated form. Subsequently the agency indicated that this contaminant is oversulfated chondroitin sulfate (OSCS), which was further confirmed by a collective group of investigators.4,5

Additional studies were needed to further characterize this substance. Although the contaminant was found to produce contact activation leading to the formation of kallikrein and the ultimate generation of bradykinin, no data on the other biological activity of this contaminant are available at this time.4 Concern also exists as to the presence of other impurities that may also be present.

**ISOLATION AND CHARACTERIZATION OF THE HEPARIN CONTAMINANT**

To further investigate the chemical and biological profiles of the contaminant in recalled unfractionated heparins (UFH), four recalled UFH preparations (three finished products and one powder) were investigated.

The contaminant in recalled heparin was isolated by removal of the heparin by heparinase-1 and/or nitrous acid depolymerization. OSCS as well as all N-acetylated galactosaminoglycans were not affected by this reaction and could be precipitated from the reaction media by ethanol. Initially, the heparin solutions (5000 U/mL vials) were lyophilized and the product was directly used in the reaction. The presence of excipients such as sodium chloride benzyl alcohol in low concentration did not affect the normal rate of the depolymerization process. The depolymerization process was repeated twice to remove all heparin fragments from the contaminant.

The amount of nondigested material in the recalled heparins ranged from 10 to 30%, most of which was characterized to be OSCS by proton and 13C-NMR spectroscopy. The molecular weight profile was determined by using size exclusion chromatography.6,7 Fig. 1A and 1B show the molecular profile of hyper-/oversulfated chondroitin sulfate (HCS-2), isolated contaminant from the recalled heparin batches (HC), contaminant-free heparin (CFH), and their respective mixtures with heparin (HCS-2 + CFH, HC + CFH).

![Figure 1](https://example.com/figure1.png)

**Figure 1** (A) Molecular profiling of hypersulfated chondroitin sulfate, heparin, and their mixtures; high-performance liquid chromatography-ultraviolet (HPLC-UV) results. (B) Molecular profile of hypersulfated chondroitin sulfates, heparin, and their mixtures; high-performance liquid chromatography-refractive index (HPLC-RI) results. CFH, contaminant-free heparin; HC, heparin contaminant; hyper-/oversulfated chondroitin sulfate-2 (HCS-2).
HCS-2 exhibited a relatively higher molecular weight of 28.1 kd, whereas HC and CFH represented comparable molecular weights of 17.2 kd and 14.1 kd, respectively. When the contaminant was mixed at a 1:1 ratio with CFH, its molecular weight profile did not differ from that of CFH; however, when HCS-2 was mixed at the same ratio it markedly altered the molecular profile of heparin. The results in both the ultraviolet (UV) and refractive index (RI) detection by high-performance liquid chromatography (HPLC) were similar. Additional studies performed with another OSCS did not show similar results, and the molecular profiles of the mixtures were similar.

The contaminated heparin (CH) exhibited a wider dispersity index in comparison with contaminant-free heparin (CFH) with oligosaccharides ranging from 5 to 30 kd (average, 16.8 kd). In addition, a well-characterized porcine cartilage OSCS preparation with average molecular weight of 17.2 kd was used as a reference material. Although varying degrees of dermatan sulfate (high molecular weight) and other minor impurities were also detected, the OSCS appeared to be the major contaminant in the recalled heparins.

### IN VITRO ANTICOAGULANT ACTIVITIES OF THE HEPARIN CONTAMINANT

To investigate the biological properties, the isolated contaminant was profiled in routinely used anticoagulant (USP, activated clotting time [ACT], activated partial thromboplastin time [aPTT], Heptest) and antiprotease (anti-FXa, anti-FIIa) assays.

Fig. 2 shows the results of the comparative studies performed on the ACT with CFH, HCS-1, HC, and their mixtures. In the USP assay, the contaminant and OSCS exhibited a potency range of 20 to 30 USP U/mg. Both the CFH and CH produced a strong anticoagulant effect in this assay at 10 mg/mL. The HCS-1 and HC produced somewhat weaker effects on the ACT at the same concentration. A 1:1 mixture of the HCS-1 and HC with CFH produced increased anticoagulant effects that were supra-additive. The contaminants isolated from different CH preparations produced a concentration-dependent anticoagulant effect in the whole blood celite ACT and saline ACT tests but was weaker than CFH.

In the plasma-based prothrombin time (PT) assay (extrinsic coagulation system) the contaminant only exhibited very weak activity and did not affect the international normalized ratio up to concentrations of 50 μg/mL. In the other anticoagulant assays such as the plasma-based aPTT (intrinsic coagulation system) and Heptest, in comparison with CFH, the contaminant produced varying degrees of concentration-dependent anticoagulant activity (10 to 40 U/mg). In the amidolytic anti-FIIa assay, the contaminant produced a concentration-dependent inhibition of thrombin in citrated plasma (~25 U/mg). This contaminant did not exhibit any inhibition of FXa.

Studies of the HCS-1 mixed with CFH in proportions of 3, 6, 12, 25, and 50% (amount of contaminant) in plasma and whole blood revealed a supra-additive, assay-dependent effect of the contaminant on both the anticoagulant and anti-FIIa activities. At a 25% level, the contaminant produced a marked increase of the anticoagulant activity of the mixture mimicking a pharmacopeial potency of ~150 U/mg; however, this increase depended on different contaminant preparations (different batches).

Figs. 3 through 6 show the results of the mixing studies. Fig. 3 shows that HCS-1 exhibited a relatively weaker activity in comparison with heparin. Addition of the contaminant at various levels with heparin produced varying degrees of anticoagulant effects in this assay. These effects were largely additive.

Fig. 4 shows the results of similar studies with the thrombin time (TT) assay. Both heparin and HCS-1 produced concentration-dependent anticoagulant effects; however, HCS-1 exhibited much weaker effects. In the mixing studies a stronger interaction between the HCS-1 and heparin was noted in this assay. At a 1:1 ratio the mixture of heparin with HCS-1 even showed a higher anticoagulant effect. The anticoagulant
effects as measured in this test were concentration dependent.

Fig. 5 shows the results of the mixing studies in the amidolytic anti-FIIa assays. In these assays the contaminant produced a concentration-dependent anti-FIIa effect with an apparent IC$_{50}$ of ~10 µg/mL, whereas heparin produced similar effects at < 2 µg/mL. At a 1:1 mixture of the HCS-1 and heparin, the IC$_{50}$ was almost the same as for heparin, indicating supra-additive interactions in this assay. The different mixtures exhibited different degrees of inhibition in this assay that were supra-additive.

As shown in Fig. 6, HCS-1 itself does not exhibit any anti-FXa effect, whereas heparin produced a concentration-dependent anti-FXa effect with an apparent IC$_{50}$ of 3 µg/mL. The mixtures of HCS-1 and heparin exhibited a stronger interaction than in other assays as measured by the IC$_{50}$ values, which were < 5 µg/mL for all mixtures. These studies clearly show that the interaction between HCS-1 and heparin are variable and depend on the type of assay used.

To further investigate the relative effects of the contaminant, commercially available antithrombin (AT) and HCII-depleted plasma preparations were supplemented in graded amounts with the contaminant and heparin. As shown in Fig. 7, in the AT-depleted plasma, whereas the anticoagulant activities of CFH were considerably reduced, the contaminant exhibited measurable concentration-dependent effects indicating a non-AT dependence. In HCII-depleted plasma, the contaminant lost sizable anticoagulant and anti-FIIa effects. The HCII activity of pre- and postheparinase digested contaminant was not different, whereas CFH showed a considerable decrease.

Table 1 shows the HCII and AT affinity profiles of HCS and HC as measured by affinity

Figure 3  Anticoagulant effects of various mixtures of unfractionated heparin (UFH) and hyper/oversulfated chondroitin sulfate (HCS) in normal human plasma as measured in the activated partial thromboplastin time (aPTT) assay.

Figure 4  Anticoagulant effects of various mixtures of unfractionated heparin (UFH) and hyper/oversulfated chondroitin sulfate (HCS) in normal human plasma as measured by the thrombin time clot-based assay.
chromatography. Whereas heparin shows sizable affinity to both HCII and AT, the HCS-1, HCS-2, and HC showed minimal affinities (<5%) to AT. Therefore, the anticoagulant effects and anti-FIIa effects of the contaminant and OSCS are mediated via HCII.

Initial studies performed on the isolation of the contaminant from heparin revealed that it was resistant to heparinase-1. Heparinase-1 and chondroitinases from IBEX Company (Montreal, Canada) and Sigma (St. Louis, MO) were used to digest the contaminant and related agents. Table 2 shows that the contaminant and the HCS preparations were resistant to heparinase-1 and chondroitinases digestion. Even extended incubation of the contaminant and OSCS with these enzymes did not result in any digestion, whereas heparin was almost completely digested into disaccharide and tetrasaccharide components by heparinase-1.

Protamine sulfate neutralized HC, HCS, CFH, and CH effectively as measured by the clot-based ACT, aPTT, and TT tests. The contaminant and HCS were also readily neutralized by polybrene and PF4 in a similar fashion as CFH. The relative degree of neutralization profile varied with each of the agents.

To study the effect of CH, CFH, HCS, and HC on the generation of kallikrein, plasma samples were supplemented in graded amounts with each of the agents and incubated with a specific substrate. Fig. 8 shows the results on the generation of kallikrein as measured by monitoring the hydrolysis of a chromogenic substrate specific for this enzyme. In addition, contact factor activation mediated by various agents was also studied. The contaminant and HCS preparations produced a relatively stronger generation of kallikrein in comparison with CFH and CH.
The effect of the contaminant and OSCS on heparinase-1 inhibition was studied by monitoring the depolymerization index using HPLC. Graded amounts of each of the agents were supplemented to a heparin substrate, which was then subjected to depolymerization by heparinase-1. Table 3 shows the results on the inhibition of heparinase-1 by the two HCS preparation and HC. All agents produced a similar inhibitory action on this enzyme with the IC50 ranging from 0.24 to 0.31 µg/mL. To study this inhibitory effect, CFH was used as a substrate.

Fig. 9 shows the results on the contaminant-mediated HIT antibody (heparin-induced thrombocytopenia) activation of platelets. In contrast to heparin, HC had a faster onset of action and longer lasting time course of platelet aggregation. In the standard 14C-serotonin release assay, the contaminant produced strong platelet activation as measured by the release of radiolabeled serotonin, which was sustained at high concentrations and did not follow the parabolic response observed with heparin.

Preliminary studies suggest that the contaminant may also exhibit direct antiprotease effects by complexing with FVIIa and the prothrombinase complex. Similar augmentation of the effects of CFH were noted with thrombin and FXa generation as measured by amidolytic methods and measurements of such thrombin generation markers as fibrinopeptide A (FPA), thrombin-antithrombin complex (TAT), and prothrombin fragment F1.2. Similar studies performed on two semisynthetic OSCS preparations mixed with noncontaminated heparin provided comparable results in the whole blood and plasma in vitro studies.

IN VIVO ANTITHROMBOTIC ACTIVITIES OF THE HEPARIN CONTAMINANT
To study the in vivo effect of the contaminant on antithrombotic activities, the effect of contaminated heparin (CH) and a potency equivalent CFH preparation were studied in established animal models of bleeding and thrombosis. A rat tail bleeding model

Table 1 Heparin Cofactor II and Antithrombin Affinity Profiles of Hypersulfated Chondroitin Sulfate and Heparin Contaminant

<table>
<thead>
<tr>
<th>Agent</th>
<th>HII</th>
<th>AT</th>
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<tbody>
<tr>
<td>HCS-1</td>
<td>32%</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>HCS-2</td>
<td>26%</td>
<td>28%</td>
</tr>
<tr>
<td>Heparin</td>
<td>26%</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

AT, antithrombin; HCS, hypersulfated chondroitin sulfate; HC, heparin contaminant.

Table 2 Biochemical Profile of Hypersulfated Chondroitin Sulfate and Heparin Contaminant*

<table>
<thead>
<tr>
<th>Agent</th>
<th>Protamine Neutral</th>
<th>Heparinase-1 Digest</th>
<th>Chondroitinase Digest*</th>
</tr>
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<tbody>
<tr>
<td>HCS-1</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HCS-2</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Heparin</td>
<td>+</td>
<td>+</td>
<td>+/</td>
</tr>
<tr>
<td>HC</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Chondroitinase A, B, C. The neutralization/digestion was measured using clot-based assays such as the activated clotting time, activated partial thromboplastin time, and thrombin time. HCS, hypersulfated chondroitin sulfate; HC, heparin contaminant.
was used to investigate the comparative bleeding effects of CFH and CH. A jugular vein clamping model and a laser-mediated microvascular thrombosis model were used to study the antithrombotic effects of CFH and CH.

As shown on Table 4, in comparison with the CFH, at an identical dosage of 2 mg/kg and 5 mg/kg the CH produced a marked increase in bleeding that was more pronounced at the higher dosage.

Similarly, the antithrombotic effect was stronger with the contaminated preparation than with heparin in the laser model of microvascular thrombosis as shown in Table 5. The contaminated heparin also exhibited stronger antithrombotic effect than heparin in a jugular vein clamping model in rats. The composite results are shown in Table 6. The contaminated heparin consistently produced stronger antithrombotic effects than CFH.

Blood pressure measurements produced variable effects in which both the contaminated and contaminant-free heparin preparations exhibited a hypotensive response in some rats.

Figure 8 Comparative effects of hypersulfated chondroitin sulfate-1 and -2 (HCS-1 and HCS-2) preparations, heparin contaminant (HC), contaminated heparin, and uncontaminated heparin in an assay for kallikrein generation. OD, optical density.

Table 3 Heparinase-1 Inhibition of Hypersulfated Chondroitin Sulfate and Heparin Contaminant*

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCS-1</td>
<td>0.24 µM</td>
</tr>
<tr>
<td>HCS-2</td>
<td>0.31 µM</td>
</tr>
<tr>
<td>HC</td>
<td>0.28 µM</td>
</tr>
</tbody>
</table>

*Contaminant-free unfractionated heparin was used in these studies as a substrate. Graded amounts of contaminant and hypersulfated chondroitin sulfate were used to determine the IC50.

HCS, hypersulfated chondroitin sulfate; HC, heparin contaminant.

Figure 9 Interactions of oversulfated chondroitin sulfate (OSCS) with heparin-induced thrombocytopenia (HIT) antibodies as measured by the 14C-serotonin release assay. BC, B-contaminant; CH, contaminated heparin; CS, chondroitin sulfate; LC, L-contaminant; NHS, normal human serum; unfractionated heparin (UFH); VC, V-contaminant.
Table 5  A Comparison of the Antithrombotic Actions of Contaminated and Noncontaminated Heparins in a Rat Model of Mesenteric Arteriole Thrombosis

<table>
<thead>
<tr>
<th>Agent</th>
<th>No. Laser Shots</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFH</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>Contaminated heparin</td>
<td>5.4 ± 0.9</td>
</tr>
<tr>
<td>Saline</td>
<td>2.6 ± 0.4</td>
</tr>
</tbody>
</table>

*All agents were tested at 2 mg/kg intravenous.
HCS, hypersulfated chondroitin sulfate; HC, heparin contaminant.

In contrast to the CFH, the contaminated preparation produced a stronger release of tissue factor pathway inhibitor (TFPI) in the rats.

**DISCUSSION**

The main contaminant in recalled heparin, OSCS was present as a heterogeneous mixture (mean molecular weight, 16.8 kd). A significant amount of dermatan sulfate (up to 10%) and chondroitin sulfate (up to 25%) were also found. It is likely that the relative amounts of OSCS and other impurities may vary in different batches of recalled heparins.

OSCS exhibited modest anticoagulant activity (20 to 40 USP U/mL) in the PT, aPTT, ACT, anti-IIa, and USP assays (without anti-FXa activity). However, OSCS mixed with heparin produced a strong interaction resulting in a disproportionately high anticoagulant activity. A 50% mixture of OSCS with heparin resulted in 100 to 190 USP U/mL (assay dependent). OSCS produced strong interaction with PF4 and was neutralizable by protamine sulfate. Contaminated heparin, CFH, and OSCS produced concentration-dependent activation of the contact system, resulting in the generation of kallikrein and bradykinin. In animal models, contaminated heparin produced stronger bleeding and antithrombotic actions in comparison with CFH. The stronger in vivo effects of the contaminated heparin are suggestive of additional complex pharmacodynamic interactions of the contaminant with heparin. These include the release of TFPI and other mediators and pharmacokinetic modulation.

Heparin is a biological product of animal origin, and the likelihood of carryover impurities during the manufacturing process is relatively high. However, improved methods have continued to provide standardized heparin preparations that have been used globally for several decades. Although chondroitin sulfate may be present in some heparin products, the OSCS is not found in heparin or in any related products. Moreover, the mammalian mast cells are incapable of producing this unnatural material. The contaminant isolated from recalled heparins is comparable to the semisynthetic OSCS and its presence can be readily identified by using physicochemical methods such as NMR.

Although the biological effects of the heparin contaminant are not fully understood at this time, these produce measurable anticoagulant effects that are somewhat weaker than heparin. However, when mixed in different proportions with heparin, the heparin contaminant produces supra-additive effects in the anticoagulant and antiprotease assays. In addition, the in vivo antithrombotic and hemorrhagic effects of the heparin contaminant and its mixture with heparin are also supra-additive.

The supra-additive effect both in vitro and in vivo may be due to the stronger affinity of the contaminant to plasma proteins, thereby releasing bound heparin from plasma proteins. Mixing of low amounts of exogenous OSCS to heparin with a molecular weight similar to heparin did not change the molecular profile of heparin, and its interaction with heparin resulted in relatively equivalent anticoagulant effects. Therefore, in routine chemical and biological methods used for heparin characterization, the contaminant remained undetectable until the adverse events came to light.

Because the contaminated batches of heparin also contain variable amounts of dermatan sulfate and potentially other impurities, additional studies are needed at

Table 6  A Comparison of the Antithrombotic Effects of Contaminated and Noncontaminated Heparins in a Rat Model of Jugular Vein Clamping

<table>
<thead>
<tr>
<th>Agent</th>
<th>Jugular Vein Clamping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.3 ± 0.4</td>
</tr>
<tr>
<td>Contaminated</td>
<td></td>
</tr>
<tr>
<td>2 mg/kg</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>7.6 ± 0.8</td>
</tr>
<tr>
<td>Noncontaminated</td>
<td></td>
</tr>
<tr>
<td>2 mg/kg</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>6.0 ± 0.4</td>
</tr>
</tbody>
</table>
this time on the pharmacological profile of the contaminated heparin, isolated contaminant, semisynthetic OSCS, hypersulfated dermatan sulfate, and their precursors. Such studies will be helpful in the understanding of the interactions of naturally occurring impurities and unnatural additives purposefully mixed with the polypharmacological heparin.

It is not uncommon to have contaminants in biological products, especially those agents used in the management of coagulation disorders. However, with the use of advanced technology and integrated collaboration with regulatory, academic, and industrial laboratories, complex biological products such as heparin can be better quality assured to provide uniform products consistently. The chemistry of heparin is complex, which lends itself to developing specific heparin fractions and components with characteristic biological properties, a principle that has been used to develop novel antithrombotic agents. However, the advanced knowledge of the structure–activity relationship of sulfated glycosaminoglycans can be misused to develop adulterated heparin products that lead to the unexpected adverse events and deaths such as reported earlier this year.

It is also important to comment that the supply of heparin largely depends on livestock. The supply and demand ratio for heparin and heparin-related products has become imbalanced in the past few years due to the expanded use of low molecular weight heparins. Moreover, issues such as infection in the animals used may compromise their health which, in itself, can reduce the supply or modify the composition of heparin. Thus, if there is a shortage of heparin, we must have heparin substitutes for the management of patients needing this important drug. Direct thrombin inhibitors such as argatroban have been used in the anticoagulant management of heparin-compromised patients. Because of the synthetic nature of argatroban and related drugs, a supply and demand issue does not exist. Therefore, it is important to develop synthetic alternate drugs for the anticoagulant management of patients in the event of a heparin shortage.

REFERENCES