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## Mass Balance Analysis of Contaminated Heparin Product

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### Abstract

A quantitative analysis of a recalled contaminated lot of heparin (HP) sodium injection United States Pharmacopeial (USP) was undertaken in response to the controversy regarding the exact nature of the contaminant involved in the HP crisis. A mass balance analysis of the formulated drug product was performed. After freeze-drying, a 1 ml vial for injection afforded  $54.8 \pm 0.3$  mg of dry solids. The excipients, sodium chloride and residual benzyl alcohol, accounted for  $11.4 \pm 0.5$  mg and  $0.9 \pm 0.5$  mg, respectively. Active pharmaceutical ingredient (API) represented  $41.5 \pm 1.0$  mg, corresponding to 75.7 wt% of dry mass. Exhaustive treatment of API with specific enzymes, heparin lyases and/or chondroitin lyases was used to close mass balance. HP represented  $30.5 \pm 0.5$  mg, corresponding to 73.5 wt% of the API. Dermatan sulfate (DS) impurity represented  $1.7 \pm 0.3$  mg, corresponding to 4.1 wt% of the API. Contaminant,  $9.3 \pm 0.1$  mg corresponding to 22.4 wt% of API, was found in the contaminated formulated drug product. The recovery of contaminant was close to quantitative (95.6-100 wt%). A single contaminant was unambiguously identified as oversulfated chondroitin sulfate (OSCS).

### Keywords

heparin; glycosaminoglycan; contaminant; oversulfated chondroitin sulfate; OSCS; dermatan sulfate; mass balance analysis; heparin lyases

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## Introduction

Heparin (HP), an anticoagulant, is used to decrease the clotting ability of the blood in the veins, arteries, or lungs and helps prevent harmful clots from forming in the blood vessels before surgery [1, 2]. HP is a natural product extracted from animal tissues, most commonly from porcine intestine, in a scale of over 100 tons per year worldwide [3] and over the past decade there has been a growing shortage of raw material on the international market. HP is a glycosaminoglycan (GAG) and is recovered from tissues as a microheterogeneous (multiple chain sequences), polydisperse (multiple chain lengths) collection of linear anionic polysaccharides chains. The most common disaccharide unit (60% – 90%) in HP is 2-*O*-sulfo- $\alpha$ -L-iduronic acid 1 $\rightarrow$ 4 glycosidically-linked to 6-*O*-sulfo-*N*-sulfo- $\alpha$ -D-glucosamine,  $\rightarrow$ 4) IdoA2S (1 $\rightarrow$ 4) GlcNS6S (1 $\rightarrow$  [4]. The remaining 10 – 40% of HP structure is comprised of as many as 7 – 10 other disaccharide units [5].

Owing to its complex structure, high molecular weight ( $MW_{avg} \sim 10,000 - 20,000$ ) charge ( $\sim -75 - -100$ ), and the absence of strong chromophores, common analytical methods often do not work well for HP. Before 2008, most manufacturers relied on coagulation assays set forward in the pharmacopeial compendia for characterization of HP and assessment of its purity. Unfortunately, there are other common GAGs in tissues that contain HP including chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS), as well as many other natural and synthetic polyanionic polysaccharides and polymers having modest to high anticoagulant activity [6 - 8]. Because of the absence of more effective quality control tests, in late 2007 and early 2008, HP became the subject of a crisis in the clinical and pharmaceutical world [3]. This crisis was attributed to a high content of an oversulfated chondroitin sulfate (OSCS, referred to throughout this manuscript as a GAG or modified GAG), identified as the primary contaminant and presumed adulterant in suspect lots of HP [9, 10]. OSCS, or persulfonated chondroitin, has the structure  $\rightarrow$ 4)  $\beta$ -D-GlcA2S, 3S(1 $\rightarrow$ 3)  $\beta$ -D-GalNAc4S, 6S(1 $\rightarrow$ , where GlcA is glucuronic acid and GalNAc is *N*-acetylgalactosamine. OSCS acts by activation of the contact system [11] and resulting in the release of bradykinin [11–13], and impacts complement activation, blood coagulation [14], fibrinolysis [15] and growth factor signaling [16]. In addition to OSCS, DS, a known impurity of HP, was also found in these suspect lots of HP [9, 10]. The U.S. Food and Drug Administration (FDA) introduced capillary electrophoresis (CE) and proton nuclear magnetic resonance (NMR) spectroscopy screening tests in response to this public health crisis [17, 18] and this was followed by a compendial revision in the USP and other pharmacopeia requiring high performance liquid chromatography (HPLC) and NMR analyses together with updated bioassays [19].

During the past two years several academic and government laboratories have been actively developing new approaches for the analysis of HP API, formulated product, low molecular weight heparins [8] and heparinized devices [20]. The separation-based analysis of HP for the presence of contaminant was based on anion-exchange HPLC [21–25], and capillary, membrane and gel electrophoresis [9, 18, 26–31]. Bioassay methods have been applied to detect OSCS in HP [32]. Spectroscopically based analysis has relied on NMR [8–10, 18, 22, 24, 33–35], infrared and Raman [36], fluorescence [37] and potentiometry [38].

Despite these major analytical advances many focus simply on ruling out the presence of OSCS in HP and with the exception of NMR, requiring a higher level of skill to use and interpret, may not detect other potential contaminants and adulterants equally well [8]. For example, retention and migration times of the broad peaks observed in HPLC and CE are profoundly impacted by changes in the  $MW_{avg}$  and charge density of the polyanion contaminant and the presence of salts in the sample.

Our laboratory pioneered the isolation, characterization and development of heparin lyases as analytical tools [39]. Heparin lyases are well-characterized endolytic enzymes that act on HP and HS. These highly specific, commercially available enzymes fail to act on other GAGs, polyanions and even HP or HS having unnatural modifications. In the current study, we relied on the high specificity of these heparin lyases, together with the related chondroitin lyases, for the quantitative analysis of a contaminated HP sample.

There has been some continuing controversy surrounding the exact nature of the contaminants found within HP drug products during the HP crisis of 2007-2008 [16, 40]. It has been speculated that OSCS was not the sole contaminant in HP [16, 41]. Systematic studies of multiple HP samples collected in Europe and US post crisis have provided conflicting results with European heparins showing exclusively OSCS contaminant and DS and solvent/salt impurities [42] and US heparins suggested to contain additional oversulfated GAG contaminants [16, 41]. Unfortunately, neither study provides details on the individual samples being tested, suggesting that some may have never made it to market or may have been adulterated after the HP crisis had ended. In an effort to clarify the identity and amount of the contaminant(s) and impurity(ies) present in the formulated HP product immediately related to the HP crisis, we have undertaken to analyze the mass balance analysis of contaminated lot of HP sodium injection USP (5000 U/ml) pulled from a hospital formulary as the result of voluntary product recall in mid-January 2008 [12].

## Materials and methods

### Materials

The contaminated formulated HP used in this study was Heparin sodium injection vials (manufactured by Baxter Healthcare Inc. USP, two boxes of 25 × 1 ml vial, 5000 USP units/ml, LOT. 117050, EXP. 11/2009) (see Figure S1 in Supplemental materials) that were voluntarily removed from use in January 2008. These vials were unopened and sealed and were kept under lock and individually dispensed for the current study. Heparin sodium from porcine intestine (160 U/mg lot # PH-58606), chondroitin sulfate A (CS-A) from bovine trachea (lot # CS-19988) and dermatan sulfate (DS) from porcine intestine (lot # DS-10596) were purchased from Celsus Laboratories, Cincinnati, Ohio. Polyacrylamide, ammonium bicarbonate, alcian blue dye, 2-cyanoacetamide and tetra-n-butylammonium hydrogen sulfate were from Sigma (St. Louis, MO). Vivapure MAXI Q columns were from Sartorius Stedim Biotech (Bohemia, NY). Membrane centrifuge filtration units (YM-3, molecular weight cut-off (MWCO) 3K) were from Millipore (Billerica, MA). HP/heparan sulfate unsaturated disaccharides standards (0S,  $\Delta$ UA-GlcNAc (where  $\Delta$ UA is  $\Delta$ -deoxy-L-threo-hex-4-enopyranosyl uronic acid); NS,  $\Delta$ UA-GlcNS; 6S,  $\Delta$ UA-GlcNAc6S; 2S,  $\Delta$ UA2S-GlcNAc; 2SNS,  $\Delta$ UA2S-GlcNS; NS6S,  $\Delta$ UA-GlcNS6S; 2S6S,  $\Delta$ UA2S-GlcNAc6S; NS2S6S,  $\Delta$ UA2S-GlcNS6S), chondroitin/dermatan sulfate unsaturated disaccharides standards (0S,  $\Delta$ UA-GalNAc; 2S,  $\Delta$ UA2S-GalNAc; 6S,  $\Delta$ UA-GalNAc6S; 4S,  $\Delta$ UA-GalNAc4S; 2S6S,  $\Delta$ UA2S-GalNAc6S; 4S6S,  $\Delta$ UA-GalNAc4S6S; 2S4S,  $\Delta$ UA2S-GalNAc4S and 2S4S6S,  $\Delta$ UA2S-GalNAc4S6S), and chondroitinase ABC (from *Proteus vulgaris*) and chondroitinase ACII (*Arthrobacter aurescens*) were obtained from Seikagaku Co. (Tokyo, Japan). Recombinant *Flavobacterium* heparin lyase I, II, and III (expressed in *E. coli*) were prepared as previously described [43]. Oversulfated chondroitin sulfate was prepared according to a procedure of Maruyama *et al.* [7]. Tributylamine (TrBA), hexylamine (HXA), 1,1,1,3,3,3-hexafluoro isopropanol (HFIP) were purchased from Sigma-Aldrich (St. Louis, MO) and were the highest purity available. Bio-Gel P-2 and P-6 gels were purchased from Bio-Rad (Hercules, CA).

## Procedures of mass balance analysis on the contaminated HP

The flow chart for the mass balance analysis of contaminated HP formulated drug product is shown in Figure 1. The major steps included: 1) desalting contaminated HP drug product using a P-6 column; 2) heparin lyase I, II and III digestion of salt free GAG and removal of heparin lyases using a strong cation exchange (SCX) spin column and the separation of HP disaccharides and buffer salts from undigested GAGs using a P-6 column; 3) chondroitin lyase ABC and chondroitin lyase ACII digestion of CS/DS and removal of chondroitin lyases using a strong cation exchange (SCX) spin column and the separation of CS/DS disaccharides and buffer salts from undigested GAGs using a P-6 column. After each steps, GAG samples were subjected to carbozole assay (against a standard curve of the same GAG) or direct mass measurement using analytical balance after freeze-dried. Structural information was collected on the isolated GAGs using NMR, polyacrylamide gel electrophoresis (PAGE), HPLC and CE. Information of the purity of HP was also obtained from disaccharide analysis by HPLC-MS.

### HPLC analysis of GAGs [21] and benzyl alcohol excipient

HPLC relied on a Shimadzu SPD-20A liquid chromatograph equipped with a UV/VIS detector. Strong anion exchange (SAX)-HPLC column is Waters Spherisorb® S5 SAX (250 mm × 4 mm) column (Waters). The chromatographic conditions for the detection of GAGs were as follows: the mobile phase solution A was distilled water and solution B was 2.5 M sodium chloride with 20 mM Tris adjusted to pH 3 by addition of phosphoric acid. The gradient was 0 – 2 min 95% A with 5% B, linear gradient to 100% B at 26 min, hold at 100% B until 31 min, linear gradient to 95% A with 5% B at 32 min and hold until end of run at 40 min. The flow rate was constant at 0.8 ml/min. UV detector was set at 215 nm. A 20- $\mu$ l injection volume for each sample at a concentration of 10 mg/ml was used. The chromatographic conditions for the detection of benzyl alcohol were as follows: the mobile phase solution A was distilled water and solution B was 2.5 M sodium chloride adjusted to pH 3 by addition of phosphoric acid. The gradient was 0 – 10 min 95% A with 5% B, linear gradient to 20% B at 20 min and 50% B at 50 min, hold at 50% B until 60 min, linear gradient to 95% A with 5% B at 61 min and hold until end of run at 70 min. Flow rate was constant at 0.8 ml/min. UV detection was at 232 nm. A 5- $\mu$ l injection volume for each sample at a concentration of 10.4 mg/ml was used.

### CE analysis of GAGs [30]

CE detection was performed on an Agilent CE instrument (Agilent Technologies, Wilmington, DE) equipped with a UV detector operating at 200 nm (band width, 10 nm). The capillary used was fused silica with a 50  $\mu$ m internal diameter and a total length of 70 cm (Supelco Inc., Bellefonte, PA). New capillaries were rinsed with 1 M sodium hydroxide for 30 min at 50 psi followed by distilled water for 10 min at 50 psi. Background electrolyte was prepared by dissolving the desired concentration of Tris and adjusting the pH to 3.0 with phosphoric acid. The separation voltage was -30 kV. A 10-s pressure injection was used for each salt-free contaminated formulated HP drug product sample.

### Desalting procedures

GAG samples for mass balance analysis first required the complete removal of salt. Three methods were examined to identify the best desalting procedure: YM-3 (3000 MWCO) spin column chromatography; P-2 column (2.5 × 80 cm, packed with 130 g of P-2) chromatography; and P-6 column (2.5 × 90 cm, packed with 70 g of P-6) chromatography. Using a YM-3 spin column, GAGs were recovered from the membrane by centrifugal filtration (10, 000 × g) after the products were washed five-times with 200  $\mu$ l water. For desalting with P-2 and P-6 columns, three eluents (water, 0.5 M NaCl and 0.5 M NH<sub>4</sub>HCO<sub>3</sub>)

were examined to optimize recovery efficiency. Fractions were detected with UV/VIS spectrophotometer with 232 nm wavelength or by carbazole assay. Salt free GAGs, confirmed by silver nitrate detection (Precipitation following the addition of 5  $\mu$ l of 0.1 M silver nitrate to 5  $\mu$ l of sample on a clean glass plate indicates the presence of chloride ion.), were collected for mass balance and structural analysis.

### Quantification of GAGs by carbazole assay

Samples were subjected to carbazole assay [9] to quantify the amount of GAGs in each sample using a standard curve constructed from either HP or OSCS. Standard curves were prepared using multiple concentrations of GAG (0, 5, 10, 20, 30, 40, 50  $\mu$ g, in 100  $\mu$ l of water). The equations of standard curves for HP and OSCS were:  $y = 0.0221x - 0.022$ ,  $R^2 = 0.9874$  and  $y = 0.009x + 0.0078$ ,  $R^2 = 0.9883$ , respectively.

### Enzymatic digestion using heparin lyases I, II, and III, and chondroitinase ABC

A mixture of heparin lyase I, II, and III (100  $\mu$ l each of: heparin lyase I (1 m-units/ $\mu$ l), heparin lyase II (3 m-units/ $\mu$ l) and heparin lyase III (15 m-units/ $\mu$ l) or the same mixture of heparin lyase I, II, and III along with chondroitinase ABC (20  $\mu$ l of 0.2 m-unit/ $\mu$ l) were added to each vial of sample in 4 ml of 50 mM pH 7 sodium phosphate/ 100 mM NaCl buffer and incubated at 31-37  $^{\circ}$ C for 72 h [39].

### Enzyme removal after digestion

After enzymatic digestion, samples were adjusted to pH 4 using glacial acetic acid and loaded onto a Vivapure MAX SH spin column. The enzymes (all positively charged) bound to the strong cation exchange (SCX) column and the GAGs (negatively charged) run through the spin columns and were collected in the wash under centrifugal force (500  $\times$  g). Then the column was washed five-times with distilled water. GAGs were recovered by pooling the eluent and washes and freeze-dried.

### Separation of intact GAGs from disaccharides and salts

The resulting protein free samples were dissolved in the 400  $\mu$ l of distilled water and loaded onto a P-6 column (2.5  $\times$  90 cm) that was pre-equilibrated in 0.5 M  $\text{NH}_4\text{HCO}_3$ . The column was eluted with 0.5 M  $\text{NH}_4\text{HCO}_3$ . Fractions (monitored at 232 nm) were collected and freeze-dried for mass analysis.

### NMR analysis

Intact GAGs, isolated from the contaminated HP drug product using P-6 column, were analyzed by  $^1\text{H}$ -NMR or 2D NMR (HMQC and HHCOSY) spectroscopy to characterize their structures. All NMR spectra were performed on Bruker 600 spectrometer with Topspin 2.0 software. Commercial DS and synthetic OSCS as well as the isolated GAGs were each dissolved in 0.5 ml  $\text{D}_2\text{O}$  (99.996%, Sigma, Co.) and freeze-dried repeatedly to remove the exchangeable protons. The resulting samples were re-dissolved in 0.3 ml  $\text{D}_2\text{O}$  and transferred to NMR microtubes (OD 5 mm, Shigemi). The operation conditions for one-dimensional  $^1\text{H}$  spectra were as follows: 256 to 512 scans, sweep width of 12.3 kHz, acquisition time of 2.66 s, and relaxation delay of 8.00 s. The conditions for 2D HMQC spectra were as follows: 32 scans, sweep width of 6.15 kHz, acquisition time of 0.33 s, and relaxation delay of 0.90 s. The conditions for 2D  $^1\text{H}$ - $^1\text{H}$ -COSY spectra were as follows: 16 scans, sweep width of 7.40 kHz, acquisition time of 0.28 s, and relaxation delay of 1.50 s.

### PAGE analysis

PAGE was used to analyze the GAGs and disaccharides recovered by P-6 column. A portion of the recovered GAG fraction (~3  $\mu$ g) was subjected to electrophoresis and compared to a

ladder of enzymatically prepared bovine lung HP oligosaccharide standards [44]. The gel was visualized with alcian blue staining and then digitized with UN-Scan-it software (Silk Scientific, Utah). The  $MW_{avg}$  of GAG, collected from P-6 column after heparin lyase and chondroitinase digestion, was calculated based on these HP oligosaccharide standards [44].

### **Disaccharide compositional analysis by HPLC-ESI-MS [45]**

The HP disaccharides, collected from P-6 column after heparin lyases digestion, were freeze-dried for LC-ESI-MS analysis. DS samples (20  $\mu\text{g}/5 \mu\text{l}$ ) were incubated with the chondroitinase ABC (10 m-units) and chondroitinase ACII (5 m-units) at 37 °C for 10 h. The enzymatic products were recovered by the centrifugal filtration (YM-3, 3000 MWCO, Millipore, Bedford, MA). DS disaccharides, passed through the filter, were freeze-dried and ready for LC-MS analysis.

The HPLC-ESI-MS analysis for disaccharides was performed on a LC-ESI-MS system (Agilent, LC/MSD trap MS). Solutions A and B for HPLC were 15 % and 70 % acetonitrile, respectively, containing the same 37.5 mM  $\text{NH}_4\text{HCO}_3$  and 11.25 mM tributylamine. The pH of solutions A and B were adjusted to 6.5 with acetic acid. Separation was performed on a C-18 column (Agilent), using solution A for 20 min, followed by a linear gradient of 0% to 50% solution B for 20 to 45 min at a flow rate was 10  $\mu\text{l}/\text{min}$ . The column effluent entered the source of the ESI-MS for continuous detection by MS. The electrospray interface was set in the negative ionization mode with the skimmer potential of  $-40.0 \text{ V}$ , capillary exit at  $-40.0 \text{ V}$ , and a source of temperature at 325 °C to obtain the maximum abundance of the ions in full scan spectra (150–1500 Da, 10 full scans/s). Nitrogen was used as a drying (5 liters/min) and nebulizing gas (20 p.s.i.).

## **Results**

### **Qualitative assessment of GAG components by HPLC and CE analysis**

An initial assessment of the components present in the contaminated HP drug product was made using SAX-HPLC chromatography [21] and CE [18]. Both HPLC and CE analyses (Figure 2 A and B) show the presence of three peaks, corresponding to HP, OSGS and DS. The identity was confirmed by co-injection.

### **Assessment of residual benzyl alcohol excipient by HPLC analysis**

The assessment of benzyl alcohol excipient in the vial of contaminated HP drug product and the residual benzyl alcohol after freeze-drying the contaminated HP drug product was made HPLC. An equation ( $y = 78.5x$ ,  $R^2 = 0.967$ ) was obtained from a standard area curve by plotting peak area obtained on analysis of aqueous benzyl alcohol solution as a function of concentration (0, 1.04 mg/ml, 2.08 mg/ml, 3.12 mg/ml, 4.16 mg/ml). There was  $10.4 \pm 0.5$  mg benzyl alcohol excipient in the vial of contaminated HP drug product, consistent with the value of 10 mg listed on the package and  $0.9 \pm 0.5$  mg of benzyl alcohol in the freeze-dried contaminated HP drug product (see Figure 2C).

### **Development of a quantitative desalting method**

Each vial of formulated HP drug product contains 7 mg sodium chloride based on the product description but additional sodium chloride can result from residual salt in the HP API and through the adjustment of formulation pH to 5.0 – 7.5 through the addition of NaOH and HCl. Thus, to accurately perform mass balance analysis on the GAGs present, the contaminated HP drug product must first be completely desalted. Three different methods were examined to find an optimal desalting procedure that allowed for complete removal of sodium chloride without loss of sample GAGs.

**Spin column (YM-3) desalting**—In the trial desalting experiment, freeze-dried artificial mixtures, containing HP (4.0/19.5 mg) and NaCl (42.2/4.3 mg) or OSCS (4.2/5.0 mg) and NaCl (42.9/46.9 mg), were in 400  $\mu$ l water were desalted using a YM-3 spin column pre-equilibrated with 200  $\mu$ l distilled water. The observed recovery efficiency (% of theoretical), of HP and OSCS, were ~78% and ~90%, respectively, (see Table S1 in Supplemental materials) suggesting this desalting method would not be useful for closing mass balance.

**P-2 column desalting**—After loading the samples (freeze-dried contaminated formulated HP drug product vials #1-6 each in 400  $\mu$ l water and freeze-dried artificial mixture of HP and OSCS as a control) on the P-2 column and washing the column with distilled water, initially eluting salt-free fractions (the absence of NaCl was confirmed by failure to form precipitate on AgNO<sub>3</sub> addition [46] and by low conductivity) were pooled and analyzed with carbazole assay. The recovery efficiency (% of theoretical) of total GAGs from contaminated formulated drug product samples and from artificial mixture of HP and OSCS, as a control, were 95.9%, 97.0 % and 82.8%, respectively (see Table S2 in Supplemental materials). The low recovery efficiency of OSCS (82.8%) is believed to be due to the adsorption of the highly negatively charged OSCS onto the P-2 resin. Because of this interaction, the GAG fraction eluted close to NaCl from the P-2 column washed with water. The total amount of NaCl was  $11.4 \pm 0.5$  mg in each contaminated formulated drug product sample, as determined using the equation ( $y = 0.0141x + 0.0361$ ,  $R^2 = 0.9901$ ) obtained from a standard conductivity curve obtained by the conductivities of aqueous NaCl solution at multiple concentrations (0, 0.1, 1, 10, 100, 1000, 10000  $\mu$ M).

**P-6 column GAG recovery/desalting**—In this procedure, salt solutions (0.5 M NaCl or 0.5 M NH<sub>4</sub>HCO<sub>3</sub>) were used as eluents to prevent the adsorption of the highly negatively charged GAGs to the P-6 resins. In GAG recovery experiments using 0.5 M NaCl or 0.5 M NH<sub>4</sub>HCO<sub>3</sub> eluents, OSCS eluted 150 ml earlier than NaCl from the P-6 column, markedly improving the resolution of the desalting step. The results (see Table 1) showed that OSCS recovery was nearly 100% on desalting using P-6 (a comparable percent recovery was observed for HP). Since NH<sub>4</sub>HCO<sub>3</sub> is a volatile buffer salt and can be easily removed by freeze-drying, in all subsequent desalting steps, P-6 column was eluted with NH<sub>4</sub>HCO<sub>3</sub>. Contaminated formulated HP drug product (vials #7-12) was desalted with this procedure and the GAG component recovered ( $41.5 \pm 1.0$  mg) was consistent with a very small deviation (see Table 1).

### Enzymatic digestion with heparin lyases and chondroitin lyases and recovery of OSCS

In a trial experiment to examine the recovery of OSCS and DS from two identical artificial mixtures of HP, OSCS and DS (#1 and #2), these mixtures were exhaustively digested with heparin lyases, the protein from enzyme digestion was removed by SCX spin columns, and #1 and #2 were subsequently separately fractionated on a P-6 column eluted with 0.5 M NH<sub>4</sub>HCO<sub>3</sub>. The recovery efficiency (% theoretical) of DS plus OSCS from the mixture was ~96% (see Table 2). In a similar trial experiment to recover OSCS from mixture of HP, OSCS and DS, two artificial mixture samples (#3 and #4) were digested with heparin lyases and chondroitin lyases, the protein from enzyme digestion was removed by SCX spin columns, and #1 and #2 were subsequently separately fractionated on a P-6 column eluted with 0.5 M NH<sub>4</sub>HCO<sub>3</sub>. The recovery efficiency (% of theoretical) of OSCS was from 95.6–100% (see Table 3). The results (Table 2 and 3) from both sets of trial experiments showed the successful recovery of OSCS or OSCS plus DS from the artificial GAG mixtures demonstrating that this method was sufficiently robust for use in mass balance analysis experiments on the contaminated formulated HP drug product. Next, six contaminated formulated HP drug product vials were used for mass balance analysis. The results (Table 2 and 3) showed that there was  $9.3 \pm 0.1$  mg of GAG, which was completely resistant to

heparin lyase and chondroitin lyase digestion. Furthermore, each vial of contaminated formulated HP drug product contained  $1.7 \pm 0.3$  mg of DS and  $30.5 \pm 0.5$  mg of HP. Chromatographic resolution of OSCS and HP disaccharides on P-6 column was excellent (Figure 3).

### NMR analysis on the recovered OSCS

The recovered OSCS from artificial GAG mixture samples and the intact heparin and chondroitin lyase-resistant GAG isolated from the contaminated HP drug product were analyzed with  $^1\text{H}$  NMR and 2D NMR ( $^1\text{H}$  NMR and HMQC see Figure 4E and Table S3 in Supplemental materials,  $^1\text{H}$ - $^1\text{H}$ -COSY see Figure S2 in Supplemental materials). The signals of intact GAG from the contaminated HP drug product, samples #10 to #12, were identical with the signals of OSCS from artificial mixtures, #3 and #4, confirming the GAG contaminant consisted of entirely OSCS. All signals of GAGs from the contaminated HP drug product, samples #7 to #9, were also consistent with those of OSCS plus DS from artificial mixtures, #1 and #2.

### PAGE analysis on the recovered OSCS

Finally, the molecular weight properties of the recovered GAG from the contaminated HP drug product were analyzed using PAGE (Figure 5). The OSCS was polydisperse with a range of MWs from 7.3-60.5 kDa and a  $\text{MW}_{\text{avg}}$  of 31.1 kDa, based on the HP oligosaccharides standards.

### Disaccharide compositional analysis

HP disaccharide composition was analyzed by HPLC-ESI-MS to provide further information on the purity of HP in the sample (Figure 6A). The percentage of the major disaccharide,  $\Delta\text{UA}2\text{S-GlcNS}6\text{S}$  (in EIC and UV, respectively) of HP recovered from the artificial mixture and contaminated HP drug product sample were 88% and 83.7%, respectively. In addition, other disaccharides, including,  $\Delta\text{UA-GlcNS}$ ,  $\Delta\text{UA-GlcNAc}6\text{S}$ ,  $\Delta\text{UA-GlcNS}6\text{S}$ ,  $\Delta\text{UA}2\text{S-GlcNS}$  and  $\Delta\text{UA}2\text{S-GlcNAc}6\text{S}$ , were detected. All the peaks in EIC spectrum were identified from their corresponding MS spectrum.

DS disaccharide composition was also analyzed by HPLC-ESI-MS (Figure 6B). The disaccharide composition profile (major and minor disaccharides) of DS isolated from contaminated HP drug product sample was well match with the commercial DS from Celsus Laboratories. The percentage of the major disaccharide,  $\Delta\text{UA-GalNAc}4\text{S}$  of DS recovered from contaminated HP drug product and commercial DS sample were 59.9% and 66.5%, respectively. From these data we can clearly conclude that the chondroitinase ABC sensitive component of the contaminated HP product is indeed DS.

## Discussion

More than two years has passed since the public health crisis caused by contaminated HP erupted. Despite numerous scientific studies from academic laboratories, industrial laboratories and government regulatory agencies, the exact identity and composition of the contaminants contained in suspect lots is still being debated [16, 40]. There are several reasons for the continued controversy. HP is a complex mixture that often contains complex impurities, such as DS. The putative contaminant, OSCS, is also a complex mixture of chains that can vary based on the  $\text{MW}_{\text{avg}}$  of its CS precursor from as well as the completeness of its chemical sulfonation [7]. The small amount of OSCS contaminant (usually reported at 1-25% [42] relative to HP makes its isolation and identification difficult. OSCS also can inhibit heparin and chondroitin lyases [47] and thus care must be take to utilize sufficient amounts of highly active enzymes in the analysis HP and DS in samples

containing OSCS. Finally, during the HP crisis there was a lack of sample control and record keeping required to definitively identify contemporaneous samples that actually were on the market in late 2007 to early 2008, administered to patients, and a cause of adverse reactions.

Thus, we undertook a study to close mass balance on Heparin sodium injection USP vials manufactured by Baxter Healthcare Inc. Two unopened boxes containing  $25 \times 1$  ml vials (LOT. 117050, EXP. 11/2009) with each vial containing 5000 USP units/ml (Figure 1) were given to our laboratory in early 2008 after they were pulled from the hospital formulary as part of the voluntary recall of suspect lots of HP. These sealed vials were kept under lock and inventory control and provided on a vial-by-vial basis to analysts in our laboratory for the current studies.

The initial GAGs qualitative assessment on the contaminated HP drug was conducted with CE and SAX HPLC by using the conditions from the references [21] and [30]. In CE analysis, the anions and cations migrate in different directions, but they are all swept towards the cathode because the electroosmotic flow (EOF) is usually significantly higher than the solute velocity due to migration (mobility). We used negative polarity in the GAG analysis. OSCS with highest negative charge was eluted first, followed by HP and then DS (see Figure 2B). SAX HPLC analysis is based on the binding affinity of GAGs to the ion exchanger containing immobilized groups of the opposite charge. The retained GAGs are eluted from the exchanger one after the other by an electrolyte gradient with increasing ionic strength. In our case, DS with lowest negative charge was eluted first, followed by HP and then OSCS (see Figure 2A). Due to the heterogeneous structure of the GAGs, it is difficult to obtain high resolution separation or to perform the qualitative analysis on these GAG mixtures. The three peaks observed corresponded to HP, DS and OSCS based on co-injection experiments (Figure 2 A and B). Unfortunately, the peaks were insufficiently resolved and too broad to obtain accurate integration and furthermore, the commercial standards (HP, OSCS and DS) came from different sources and may have afforded standard curves that were not reliable for the accurate calculation of the GAG contents in the sample.

The critical issue in closing mass balance on a formulated drug product is the complete removal of excipients while quantitatively recovering the API. According to the package insert each vial of formulated product contained 5000 USP units of HP (at 150–200 U/mg this corresponds to 25–34 mg of API), 7 mg of added NaCl, 10.44 mg benzyl alcohol, added NaOH and HCl to adjust pH to 5.0 to 7.5, made up to 1 ml with water. This corresponds to a mass on freeze-drying of 42–53 mg (assuming that some portion of the benzyl alcohol (bp 208 °C) remains). The observed mass was  $54.8 \pm 0.3$  consistent with the presence of additional components (i.e., impurity and/or contaminant) in the API. Next, series of control experiments were used to ensure the removal of the NaCl (MW 58 gm/mol) and benzyl alcohol (MW 108 gm/mol) excipients. Because of their low MW compared to GAGs in the API, our initial focus was on size separation. Three different methods including spin column (YM-3), P-2 column and P-6 column were tested using HP, OSCS and contaminated HP drug product samples. Using an artificial mixture of HP, OSCS and NaCl, the spin column (Table S1 in Supplemental materials), showed a variable recovery efficiency of from 77.5–90.5% with a higher recovery for the higher MW and higher charged OSCS than for HP. The P-2 column also has some drawbacks for the quantitative removal of salt (Table S2 Supplemental materials) showing a recovery efficiency of 82.8% for OSCS and 97% for HP. The recovery efficiency of 95.9% for HP from contaminated HP drug product samples was slightly lower because the salt elutes from the P-2 column close to the GAG. The adsorption of OSCS on P-2 column further retards its elution. Thus, simple desalting of GAG component using a P-2 column eluted with water provides insufficient resolution to be used to adequately separate GAG components from salt for closure of mass balance.

A P-6 column eluted with 0.5 M aqueous  $\text{NH}_4\text{HCO}_3$  provided the optimal method to both desalt the sample and to separate small molecules of  $\text{MW} < 6000$  (*i.e.*, salt, benzyl alcohol and disaccharides) from GAG. The presence of salt in the eluent eliminated the adsorption of negatively charged OSCS onto the polyacrylamide resin. In artificial mixtures of GAGs and salt, the recovery of GAGs was repeatedly  $>95\%$  (the total recovery efficiency after the processes of enzyme digestion, de-proteination and separation). From  $54.8 \pm 0.3$  mg of freeze-died contaminated HP drug product  $41.5 \pm 1.0$  mg of GAG was recovered (Table 2), which is again higher than the 25–34 mg of API in the sample. No GAG loss from the sample was observed using carbazole assay on the other fractions.

Enzymatic digestion studies were next employed to complete the analysis and close mass balance. The heparin lyases used were recombinant enzymes of high specific activity and were expressed in *E. coli*, an organism having no activity towards GAGs and the chondroitin lyases were purified from organisms that do not contain heparin lyases [48]. Thus, these enzymes represent ideal tools to determine GAG composition. There have been few papers reporting the analysis of contaminated HP drug product sample using enzymatic digestion since the impurity and contaminants (DS and OSCS) reportedly inhibit the heparin lyases. This inhibition however is competitive, and reversible, thus, prolonged treatment (72 h) with highly active heparin lyases at  $31\text{--}37^\circ\text{C}$  in sodium phosphate/NaCl buffer can digest completely HP [39]. Heparin lyase and heparin and chondroitin lyases (cationic proteins), used to treat the  $41.5 \pm 1.0$  mg of recovered GAG, were removed by cation exchange using a Vivapure MAXI SH column and the separation on P-6 column to afford  $11.0 \pm 0.3$  of non-HP GAG and  $9.3 \pm 0.1$  mg of non-HP, non-DS/CS GAG. These data indicate that a single vial contains  $30.5 \pm 0.5$  mg of HP (heparin lyase digestible),  $9.3 \pm 0.1$  mg of contaminating GAG (not digestible by heparin and chondroitin lyases) and  $1.7 \pm 0.3$  mg of impurity (chondroitin lyase digestible). The contaminant, resistant to heparin and chondroitin lyases was unambiguously identified as OSCS using 1D or 2D NMR ( $^1\text{H}$ NMR, HMQC and HHCOSY) (Figure 4). OSCS recovered from artificial mixtures #3 and #4 afforded spectra giving identical signals to those of the contaminant recovered from the contaminated HP drug product. Even minor signals (*i.e.*,  $\delta_{\text{H}}$  3.02 and 3.44–3.63 ppm) corresponding to impurities assigned to residual ammonium bicarbonate were the same in each sample. The  $\text{MW}_{\text{avg}}$  of OSCS from the contaminated HP drug product samples was 31.1 kDa as determined by PAGE analysis. The impurity resistant to heparin lyase digestion but sensitive to chondroitin lyase digestion was identified as DS based NMR and the presence of all the signals found in the DS recovered from the artificial mixture. The signals of DS in artificial mixture #1 and #2 at  $\delta_{\text{H}}$  1.99, 3.46 and 3.71–3.78 ppm are of greater intensity than those in the contaminated HP drug product sample #7 to #9, because of the larger amount of DS (3.7 and 4.2 mg in artificial mixtures #3 and #4, respectively) in the artificial mixtures than in the contaminated HP drug product sample ( $1.7 \pm 0.3$  mg).

HPLC-MS was applied to differentiate HP from HS, as HS was another potential impurity of HP product and can be also degraded by heparin lyases. Since HP primarily is highly sulfated, with  $\sim 60\text{--}90\%$  of the disaccharide units consisting of  $\rightarrow 4$  IdoA2S (1 $\rightarrow$ 4) GlcNS6S (1 $\rightarrow$  , on heparin lyase treatment it should give rise to primarily ( $\sim 60\text{--}90\%$ )  $\Delta\text{UA}2\text{S}$  (1 $\rightarrow$ 4) GlcNS6S (TriS). In contrast, HS is less sulfated, with  $\sim 60\text{--}90\%$  of the disaccharide units consisting of  $\rightarrow 4$   $\beta\text{-D-GlcA}$  (1 $\rightarrow$ 4)  $\beta\text{-D-GlcNAc}$ , on heparin lyase treatment it should give rise to primarily ( $\sim 60\text{--}90\%$ )  $\Delta\text{UA}$  (1 $\rightarrow$ 4) GlcNAc (OS). Disaccharide analysis of the contaminated HP drug product sample #7 to #12 isolated using P-6 column was measured (Figure 6A) showed 83.7% TriS disaccharide unit with no observable OS disaccharide, confirming that there was no detectable HS present in the contaminated HP drug product. While we cannot absolutely rule out the presence of any other contaminants or impurities (at levels below a few weight percent) in the contaminated drug product we see no indication of their presence. In particular, we see no evidence of the presence of either intact or

fragmented oversulfated DS, HP or HS. Our recovery of total GAGs (including oversulfated GAGs) from contaminated HP drug product is nearly quantitative with no residual GAGs present in our purification side streams (Figure 2) as determined by carbazole assay (having a limit of detection of <1 µg). Furthermore, our NMR analysis of recovered GAGs show no evidence of signals characteristic [10, 40] of either intact or fragmented oversulfated DS, HP or HS.

In conclusion, a mass balance analysis of a known contaminated HP product was performed. On freeze drying, the contaminated vial contained  $54.8 \pm 0.3$  mg of dry weight comprised of  $11.4 \pm 0.5$  mg of NaCl and  $0.9 \pm 0.5$  mg of residual benzyl alcohol (added excipients),  $30.5 \pm 0.5$  mg was HP,  $9.3 \pm 0.1$  mg was OSCS and  $1.7 \pm 0.3$  mg was DS. No other contaminants or impurities were detected. The detection limit of the primary assay used to close mass balance, weighing ( $\pm 0.1$  mg on an ~10 mg sample), is insignificant in our mass balance analysis compared to the recovery yields. Furthermore, the application of NMR, carbazole assay (with a limit of detection of 1 µg) and disaccharide analysis by HPLC-MS (with a limit of detection of 1 ng of disaccharide) confirmed that no significant contaminants or impurities were overlooked or missed in these analyses. This method can prospectively applied to determine many other types of potential contaminants present in HP.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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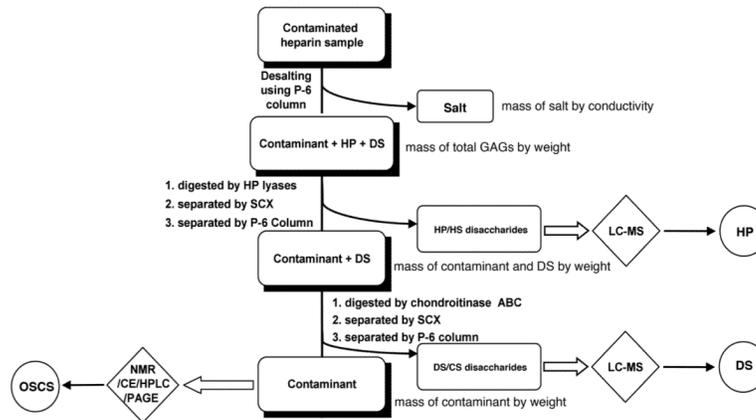
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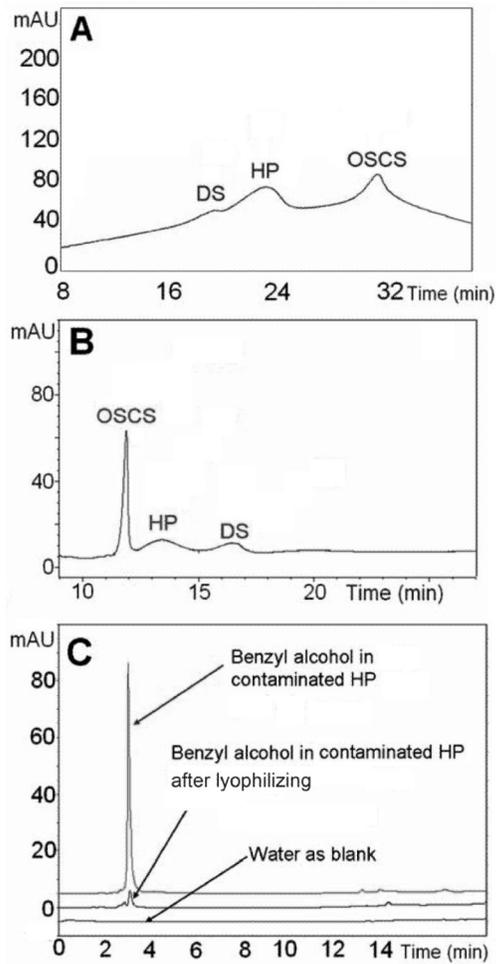
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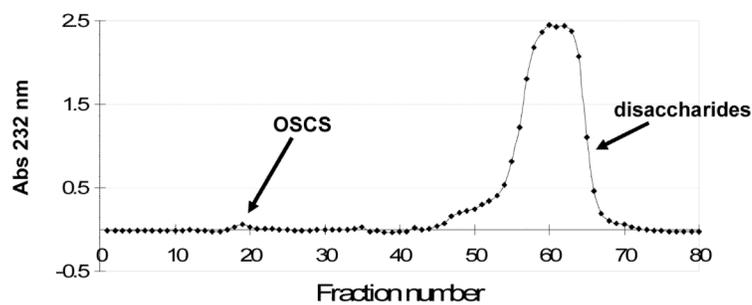
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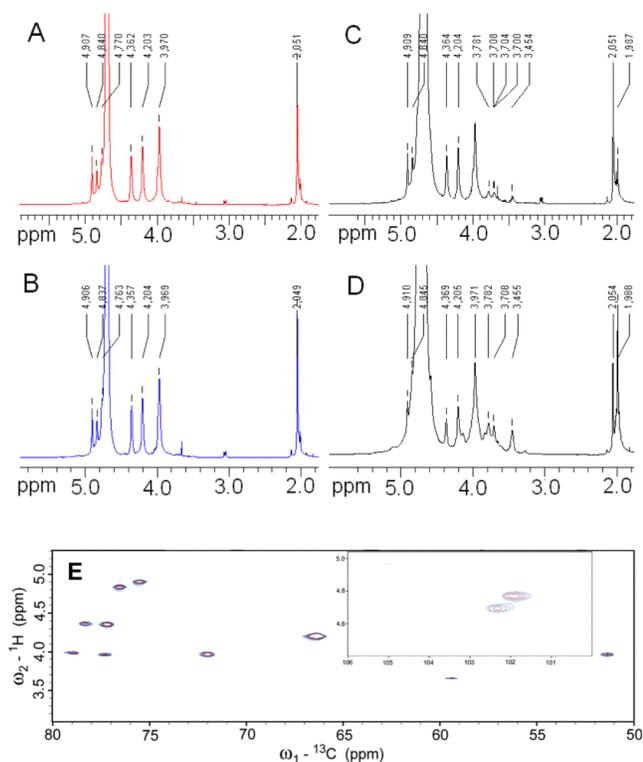
**Fig. 1.** The flow chart of mass balance analysis. First, the of contaminated HP drug product was desalted to obtain the mass of salt and the mass of total GAGs. Next, HP lyases were used to convert HP/HS to disaccharides for LC-MS analysis and the mass of contaminant and DS are determined. Finally, chondroitinase ABC was used to convert DS to disaccharides for LC-MS analysis and the mass of contaminant was determined and its composition was established as OSCS based on NMR/CE/HPLC/PAGE analysis.



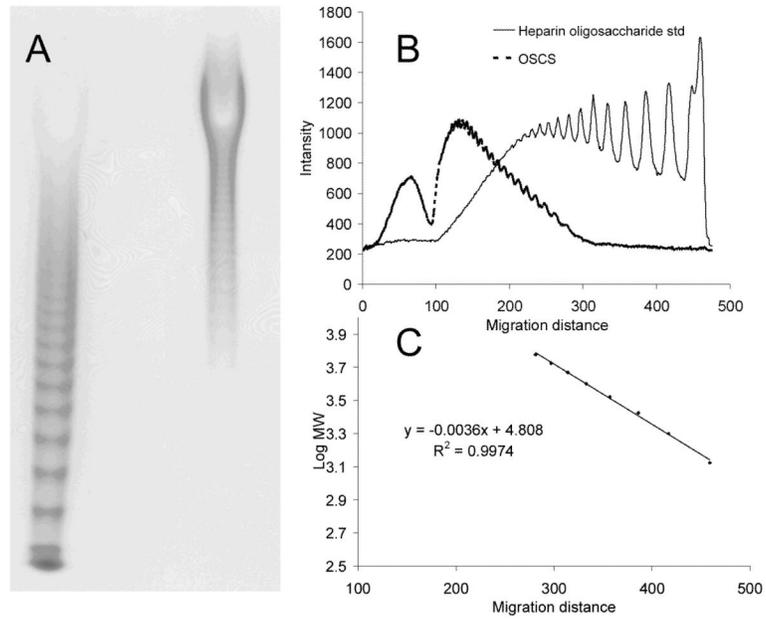
**Fig. 2.** Fractionation of contaminated HP drug product. **A.** SAX-HPLC chromatograph of contaminated HP drug product sample; **B.** CE electropherogram of contaminated HP drug product sample following desalting using P-6 column eluted with  $\text{NH}_4\text{HCO}_3$ ; **C.** HPLC chromatogram of benzyl alcohol in contaminated HP drug product sample and freeze-dried contaminated HP drug product sample.



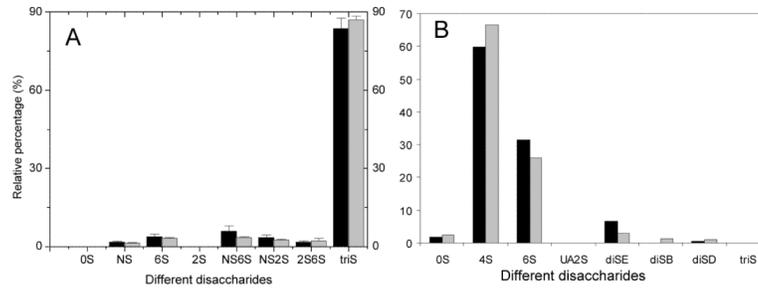
**Fig. 3.** Chromatography on P-6 column of contaminated HP drug product after processing. Contaminated HP drug product was desalted using P-6 column eluted with  $\text{NH}_4\text{HCO}_3$  and recovered GAG was freeze dried and added to PBS and treated with heparin lyase (I, II and III) and chondroitinase ABC. Enzymes were removed using SCX spin column and the sample was analyzed on P-6 with UV 232 detection. Peaks corresponding to OSCS and HP disaccharides are indicated.



**Fig. 4.** 600 MHz NMR spectra of GAG samples. **A.** Proton NMR spectrum of OSCS recovered from contaminated HP drug product. HP and DS were removed by complete digestion with heparin lyases and chondroitinase ABC; **B.** Proton NMR spectrum of OSCS recovered from artificial mixture of HP (33.9 mg), OSCS (4.5 mg) and DS (4.2 mg). HP and DS were removed by complete digestion with heparin lyases and chondroitinase ABC; **C.** Proton NMR spectrum of OSCS and DS recovered from contaminated HP drug product. HP was removed by complete digestion with heparin lyases; **D.** Proton NMR spectrum of OSCS and DS recovered from artificial mixture of HP (32.5 mg), OSCS (4.6 mg) and DS (5.0 mg). HP was removed by complete digestion with heparin lyases; **E.**  $^1\text{H}$ - $^{13}\text{C}$  HMQC spectrum for OSCS recovered from contaminated HP drug product (see panel **A**) (red) overlaid onto OSCS isolated from an artificial mixture of HP, OSCS and DS (see panel **B**) (blue),



**Fig. 5.** PAGE analysis of OSCS recovered from contaminated HP drug product. **A.** Left lane HP oligosaccharide standards; Right lane, recovered OSCS; **B.** bands intensity of HP oligosaccharide standards and recovered OSCS; **C.** A plot of log MW as a function of migration distance used to estimate the MW of OSCS contaminant.



**Fig. 6.**

**A.** Disaccharide composition of HP recovered from contaminated HP drug product (black bars) and disaccharide composition of HP recovered from an artificial mixture of HP, OSCS and DS (grey bars). **B.** Disaccharide composition of DS recovered from contaminated HP drug product sample (black bars) and commercial porcine intestinal DS from Celsus Laboratories (grey bars).

**Table 1**

The recovery efficiency (%) of OSCS and GAGs of contaminated HP drug product using P-6 column eluted with 0.5 M NaCl or 0.5 M NH<sub>4</sub>HCO<sub>3</sub>.

	OSCS <sup>a</sup>		Contaminated HP sample 7 — 12 (eluted with NH <sub>4</sub> HCO <sub>3</sub> )
	Eluted with NaCl	NaCl NH <sub>4</sub> HCO <sub>3</sub>	
Loading (mg)	2.1 ± 0.3 <sup>b</sup>	2.6 ± 0.3 <sup>b</sup>	54.8 ± 0.3 <sup>c</sup>
Recovery (mg) <sup>d</sup>	2.2 ± 0.3 <sup>b</sup>	2.6 ± 0.3 <sup>b</sup>	41.5 ± 1.0 <sup>c</sup>
Recovery efficiency (%)	105	100	-

<sup>a</sup>No salt is present in OSCS sample.

<sup>b</sup>Data represents the average values (wt) ± error of duplicate experiments.

<sup>c</sup>Data represent the average values (wt) ± SE of sextuplicate experiments.

<sup>d</sup>Recovery of OSCS was determined using carbazole assay.

Mass balance of GAGs of artificial mixture of HP, OSCS and DS (sample 1 and 2) and contaminated HP (sample 7, 8 and 9) obtained following desalting using P-6 column eluted with 0.5 M NH<sub>4</sub>HCO<sub>3</sub> and digestion with heparin lyase I, II and III.

**Table 2**

	Artificial mixture		Contaminated HP drug product
	1	2	7—9
Amount loaded (mg)	HP	32.5	24.1
	DS	5.0	3.3
	OSCS	4.6	8.3
GAG	Amount recovered (mg)	9.2	11.1
	Recovery efficiency (%)	95.8	95.7
	Calculated mass of HP (mg)	-	-
			11.0 ± 0.3*
			30.5 ± 0.5*

\* Data represent the average values (wt) ± SD of triplicate experiments.

Mass balance of GAGs of artificial mixture of HP, OSCS and DS (sample 3 and 4) and contaminated HP (sample 10, 11 and 12) obtained following desalting using P-6 column eluted with 0.5 M  $\text{NH}_4\text{HCO}_3$  and digestion with heparin lyase, I, II, and III and chondroitinase ABC.

**Table 3**

	Artificial mixture		Contaminated HP drug product	
	3	4	10	11 — 12
	HP	33.9	30.6	
Amount loaded (mg)	DS	4.2	3.7	41.5 ± 1.0*
	OSCS	4.5	6.6	
GAG	OSCS	4.3	6.6	9.3 ± 0.1*
Amount recovered (mg)	OSCS	95.6	100	-
Recovery efficiency (%)	Calculated mass of DS (mg)			1.7 ± 0.3*

\* Data represent the average values (wt) ± SD of triplicate experiment.