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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 sodium injection U.S. Pharmacopeia (USP) was undertaken in response to the controversy regarding the exact nature of the contaminant involved in the heparin (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 ± 0.3 mg of dry solids. The excipients, sodium chloride and residual benzyl alcohol, accounted for 11.4 ± 0.5 and 0.9 ± 0.5 mg, respectively. Active pharmaceutical ingredient (API) represented 41.5 ± 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 ± 0.5 mg, corresponding to 73.5 wt% of the API. Dermatan sulfate (DS) impurity represented 1.7 ± 0.3 mg, corresponding to 4.1 wt% of API. Contaminant, representing 9.3 ± 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).

Heparin (HP),[1] an anticoagulant, is used to decrease the clotting ability of the blood in the veins, arteries, or lungs and helps to 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, on a scale of more than 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 polysaccharide chains. The most common disaccharide unit (60–90%) in HP is 2-*O*-sulfo-α-L-iduronic acid 1→4 glycosidically linked to 6-*O*-sulfo-*N*-sulfo-α-D-glucosamine [→4) IdoA2S (1→4) GlcNS6S (1→, where IdoA is L-iduronic acid and GlcN is D-glucosamine] [4]. The remaining 10–40% of HP structure is composed of as many as 7–10 other disaccharide units [5].

Owing to its complex structure, high-molecular-weight (MW_{avg} ~ 10,000–20,000) charge (~−75 to −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,

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[1] *Abbreviations used:* HP, heparin; GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; OSCS, oversulfated chondroitin sulfate; FDA, U.S. Food and Drug Administration; CE, capillary electrophoresis; NMR, nuclear magnetic resonance; USP, U.S. Pharmacopeia; HPLC, high-performance liquid chromatography; CS-A, chondroitin sulfate A; MWCO, molecular weight cutoff; TrBA, tributylamine; HXA, hexylamine; HFIP, 1,1,1,3,3,3-hexafluoro isopropanol; SCX, strong cation exchange; PAGE, polyacrylamide gel electrophoresis; MS, mass spectrometry; UV/VIS, ultraviolet/visible; SAX, strong anion exchange; 2D, two-dimensional; HMQC, heteronuclear multiple quantum correlation; HHCOSY, homonuclear correlation spectroscopy; OD, optical density; 1D, one-dimensional; LC, liquid chromatography; ESI, electrospray ionization; EIC, extracted ion chromatogram; EOF, electroosmotic flow; wt, weight.

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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 article 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 →4) β-D-GlcA2S, 3S(1→3) β-D-GalNAc4S, 6S(1→, where GlcA is glucuronic acid and GalNAc is *N*-acetylgalactosamine. OSCS acts by activation of the contact system [11], resulting in the release of bradykinin [11–13], and affects 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 U.S. Pharmacopeia (USP) and other pharmacopeia requiring high-performance liquid chromatography (HPLC) and NMR analyses together with updated bioassays [19].

During the past 2 years, several academic and government laboratories have been actively developing new approaches for the analysis of HP API, formulated product, low-molecular-weight HPs [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 on capillary, membrane, and gel electrophoresis [9,18,26–30]. Bioassay methods have been applied to detect OSCS in HP [31]. Spectroscopically based analysis has relied on NMR [18,22,24,32–34], infrared and Raman [20], fluorescence [35], and potentiometry [36].

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 might 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 affected 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 [37]. 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 [16,38]. It was speculated that OSCS was not the sole contaminant in HP [16,39]. Systematic studies of multiple HP samples collected in Europe and the United States postcrisis provided conflicting results, with European HPs showing exclusively OSCS contaminant and DS and solvent/salt impurities [40] and U.S. HPs suggested to contain additional oversulfated GAG contaminants [16,39]. 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 undertook to analyze the mass balance analysis of a contaminated lot of heparin 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 USP, two boxes of 25 × 1-ml vials, 5000 USP U/ml, lot 117050, exp. 11/2009) (see Fig. S1 in supplementary material) 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 DS from porcine intestine (lot DS-10596) were purchased from Celsus Laboratories (Cincinnati, OH, USA). Polyacrylamide, ammonium bicarbonate, alcian blue dye, 2-cyanoacetamide, and tetra-*n*-butylammonium hydrogen sulfate were obtained from Sigma (St. Louis, MO, USA). Vivapure MAXI Q columns were obtained from Sartorious Stedim Biotech (Bohemia, NY, USA). Membrane centrifuge filtration units (YM-3, molecular weight cutoff [MWCO] = 3000) were purchased from Millipore (Billerica, MA, USA). HP/HS unsaturated disaccharide standards [0S, ΔUA-GlcNAc (where ΔUA is Δ-deoxy-L-*threo*-hex-4-enopyranosyl uronic acid); NS, ΔUA-GlcNS; 6S, ΔUA-GlcNAc6S; 2S, ΔUA2S-GlcNAc; 2SNS, ΔUA2S-GlcNS; NS6S, ΔUA-GlcNS6S; 2S6S, ΔUA2S-GlcNAc6S; NS2S6S, ΔUA2S-GlcNS6S], chondroitin/DS unsaturated disaccharide standards (0S, ΔUA-GalNAc; 2S, ΔUA2S-GalNAc; 6S, ΔUA-GalNAc6S; 4S, ΔUA-GalNAc4S; 2S6S, ΔUA2S-GalNAc6S; 4S6S, ΔUA-GalNAc4S6S; 2S4S, ΔUA2S-GalNAc4S and 2S4S6S, ΔUA2S-GalNAc4S6S), and chondroitinase ABC (from *Proteus vulgaris*) and chondroitinase ACII (from *Arthrobacter aurescens*) were obtained from Seikagaku (Tokyo, Japan). Recombinant *Flavobacterium* heparin lyases I, II, and III (expressed in *Escherichia coli*) were prepared as described previously [41]. OSCS was prepared according to the procedure of Maruyama and coworkers [7]. Tributylamine (TrBA), hexylamine (HXA), and 1,1,1,3,3,3-hexafluoro isopropanol (HFIP) were purchased from Sigma–Aldrich and were the highest purity available. Bio-Gel P-2 and P-6 gels were purchased from Bio-Rad (Hercules, CA, USA).

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 Fig. 1. The major steps included (i) desalting contaminated HP drug product using a P-6 column; (ii) heparin lyase I, II, and III digestion of salt-free GAGs 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; and (iii) chondroitin lyase ABC and chondroitin lyase ACII digestion of CS/DS and removal of chondroitin lyases using an SCX spin column and the separation of CS/DS disaccharides and buffer salts from undigested GAGs using a P-6 column. After each step, GAG samples were subjected to carbazole assay (against a standard curve of the same GAGs) or direct mass measurement using analytical balance after being 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–mass spectrometry (MS).

HPLC analysis of GAGs and benzyl alcohol excipient

HPLC relied on a Shimadzu SPD-20A liquid chromatograph equipped with an ultraviolet/visible (UV/VIS) detector [21]. The strong anion exchange (SAX)–HPLC column was a Waters Spherisorb S5 SAX column (250 × 4 mm). The chromatographic conditions for the detection of GAGs were as follows. The mobile phase solution

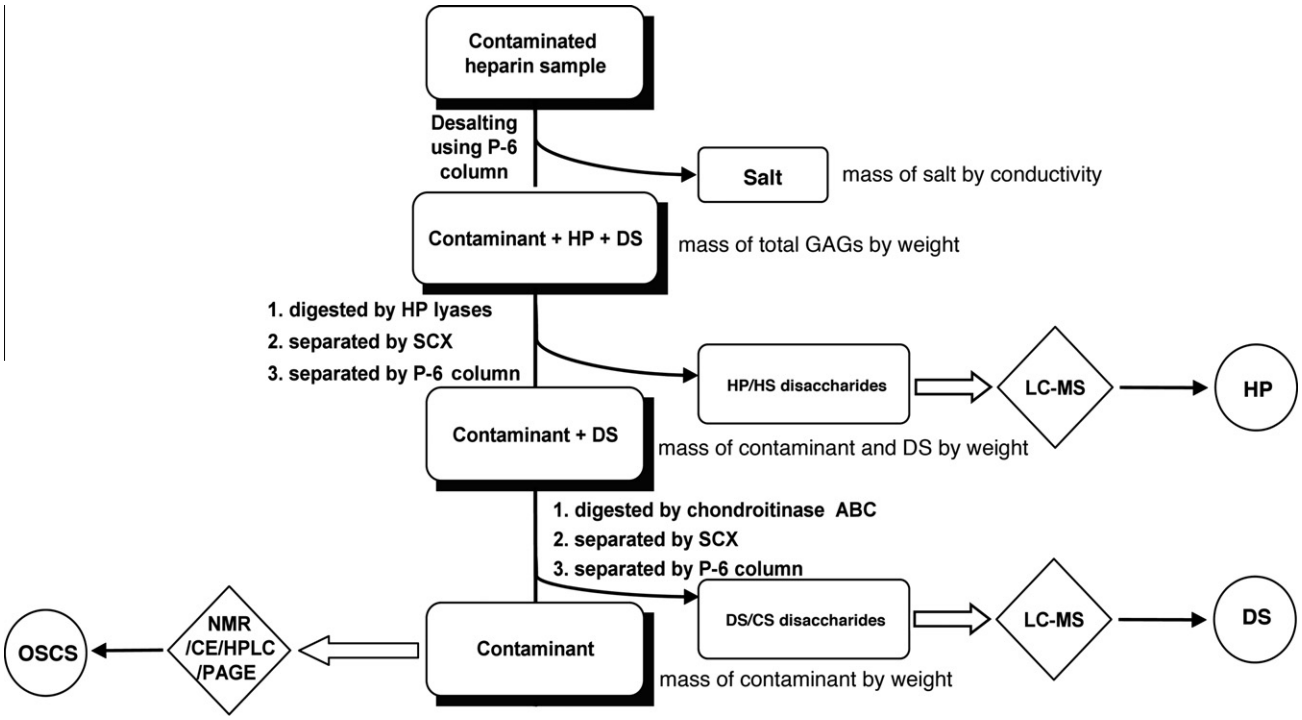

Fig.1. Flow chart of mass balance analysis. First, the contaminated HP drug product was desalted to obtain the mass of salt and the mass of total GAGs. Next, HP lyases were used to convert HP/HS to disaccharides for LC–MS analysis, and the masses of contaminant and DS were determined. Finally, chondroitinase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

A was distilled water, and solution B was 2.5 M sodium chloride with 20 mM Tris adjusted to pH 3.0 by the addition of phosphoric acid. The gradient was 0–2 min of 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. The UV detector was set at 215 nm. A 20-μl 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 3.0 by the addition of phosphoric acid. The gradient was 0–10 min of 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. The flow rate was constant at 0.8 ml/min. UV detection was at 232 nm. A 5-μl injection volume for each sample at a concentration of 10.4 mg/ml was used.

CE analysis of GAGs

CE detection was performed on an Agilent CE instrument (Agilent Technologies, Wilmington, DE, USA) equipped with a UV detector operating at 200 nm (bandwidth = 10 nm) [30]. The capillary used was fused silica with a 50-μm internal diameter and a total length of 70 cm (Supelco, Bellefonte, PA, USA). 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 spin column (MWCO = 3000) 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 μl of water. For desalting with P-2 and P-6 columns, three eluents (water, 0.5 M NaCl, and 0.5 M NH_4HCO_3) were examined to optimize recovery efficiency. Fractions were detected with a UV/VIS spectrophotometer with a 232-nm wavelength or by carbazole assay. Salt-free GAGs, confirmed by silver nitrate detection (where precipitation following the addition of 5 μl of 0.1 M silver nitrate to 5 μl 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 GAGs (0, 5, 10, 20, 30, 40, and 50 μg in 100 μl 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 lyases I, II, and III [100 μl each of: heparin lyase I (1 m-unit/μl), heparin lyase II (3 m-units/μl), and heparin lyase III (15 m-units/μl)] or the same mixture of heparin lyases I, II, and III along with chondroitinase ABC (20 μl of 0.2 m-unit/μl) was added to each vial of sample in 4 ml of 50 mM sodium phosphate/100 mM NaCl buffer (pH 7.0) and incubated at 31–37 °C for 72 h [37].

Enzyme removal after digestion

After enzymatic digestion, samples were adjusted to pH 4.0 using glacial acetic acid and were loaded onto a Vivapure MAX SH spin column. The enzymes (all positively charged) bound to the SCX column, and the GAGs (negatively charged) ran through the spin columns and were collected in the wash under centrifugal force (500*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 μl of distilled water and loaded onto a P-6 column (2.5 × 90 cm) that was preequilibrated in 0.5 M NH_4HCO_3. The column was eluted with 0.5 M NH_4HCO_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 a P-6 column, were analyzed by ^{1}H NMR or two-dimensional (2D) NMR spectroscopy (heteronuclear multiple quantum correlation [HMQC] and homonuclear correlation spectroscopy [HHCOSY]) to characterize their structures. All NMR spectra were performed on a 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 of D_2O (99.996%, Sigma) and freeze-dried repeatedly to remove the exchangeable protons. The resulting samples were redissolved in 0.3 ml of D_2O and transferred to NMR microtubes (optical density [OD] = 5 mm, Shigemi). The operation conditions for one-dimensional (1D) ^{1}H spectra were as follows: 256–512 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}H–^{1}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 a P-6 column. A portion of the recovered GAG fraction (~3 μg) was subjected to electrophoresis and compared with a ladder of enzymatically prepared bovine lung HP oligosaccharide standards [42]. The gel was visualized with alcian blue staining and then digitized with UN-Scan-it software (Silk Scientific, Orem, UT, USA). The MW_{avg} of GAGs, collected from the P-6 column after heparin lyase and chondroitinase digestion, was calculated based on these HP oligosaccharide standards [42].

Disaccharide compositional analysis by HPLC–ESI–MS

The HP disaccharides, collected from a P-6 column after heparin lyase digestion, were freeze-dried for liquid chromatography (LC)–electrospray ionization (ESI)–MS analysis [43]. DS samples (20 μg/5 μl) 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, MWCO = 3000, Millipore, Bedford, MA, USA). 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 an LC–ESI–MS system (LC/MSD trap mass spectrometer, Agilent). Solutions A and B for HPLC were 15% and 70% acetonitrile, respectively, containing the same 37.5 mM NH_4HCO_3 and 11.25 mM TrBA. The pH of solutions A and B was 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 0–50% solution B for 20–45 min at a flow rate of 10 μl/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 a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source of temperature of 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 L/min) and nebulizing (20 psi) gas.

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

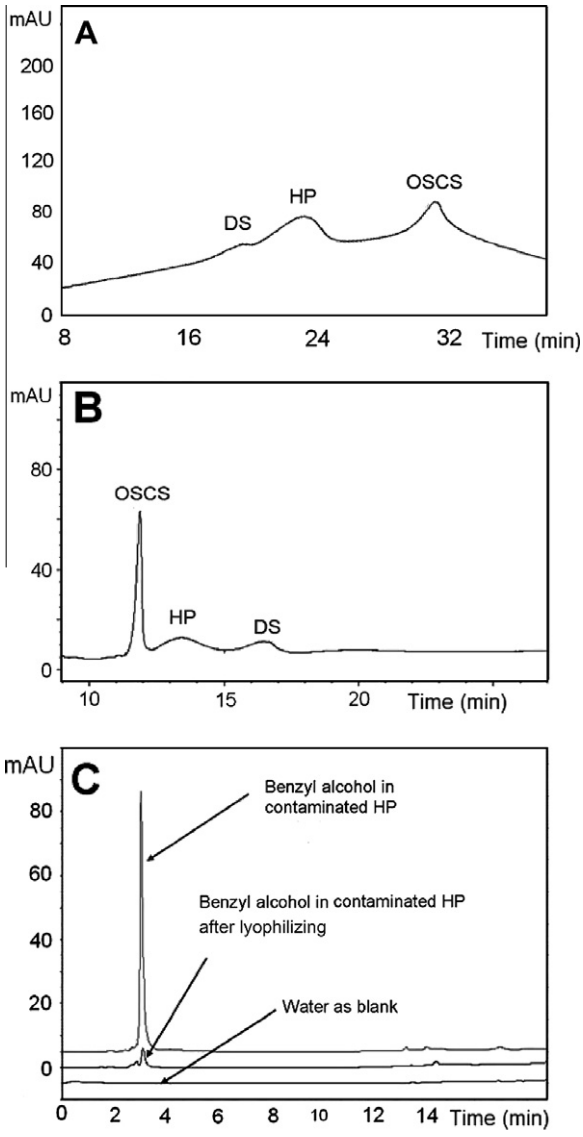

Fig.2. Fractionation of contaminated HP drug product. (A) SAX–HPLC chromatograph of contaminated HP drug product sample. (B) CE electropherogram of contaminated HP drug product sample following desalting using a P-6 column eluted with NH_4HCO_3. HPLC chromatogram of benzyl alcohol in contaminated HP drug product sample and freeze-dried contaminated HP drug product sample.

[21] and CE [18]. Both HPLC and CE analyses (Fig. 2A and B, respectively) show the presence of three peaks corresponding to HP, OSCS, and DS. The identity was confirmed by coinjection.

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 by 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, 2.08, 3.12, and 4.16 mg/ml). There was 10.4 ± 0.5 mg of 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 ± 0.5 mg of benzyl alcohol in the freeze-dried contaminated HP drug product (see Fig. 2C).

Development of a quantitative desalting method

Each vial of formulated HP drug product contains 7 mg of 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 complete removal of sodium chloride without loss of GAG samples.

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), in 400 μl of water were desalted using a YM-3 spin column preequilibrated with 200 μl of distilled water. The observed recovery efficiencies (% of theoretical) of HP and OSCS were approximately 78% and 90%, respectively (see Table S1 in supplementary material), suggesting that 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 μl of water, and freeze-dried artificial mixture of HP and OSCS as a control) on a 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 the addition of $AgNO_3$ [44] and by low conductivity) were pooled and analyzed with carbazole assay. The recovery efficiencies (% 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 supplementary material). 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 ± 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, and 10,000 μM).

P-6 column GAG recovery/desalting

In this procedure, salt solutions (0.5 M NaCl or 0.5 M NH_4HCO_3) 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_4HCO_3 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 percentage recovery was observed for HP). Because NH_4HCO_3 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_4HCO_3. Contaminated formulated HP drug product (vials 7–12) was desalted with this procedure, and the GAG component recovered (41.5 ± 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 (samples 1 and 2), these mixtures were exhaustively digested with heparin lyases, the protein from enzyme digestion was removed by SCX spin columns, and artificial mixtures 1 and 2 were subsequently separately fractionated on a P-6 column eluted with 0.5 M NH_4HCO_3. The recovery efficiency (% theoretical) of DS plus OSCS from the mixture was approximately 96% (see Table 2). In a similar trial experiment to recover OSCS from mixture of HP, OSCS, and DS, two artificial mixtures (samples 3 and 4) were digested with heparin lyases and chondroitin lyases, the protein from enzyme digestion was removed by SCX spin columns, and artificial mixtures 1 and 2 were subsequently separately fractionated on a P-6 column eluted with 0.5 M NH_4HCO_3. The recovery efficiency (% of theoretical) of OSCS was from 95.6% to 100% (see Table 3). The results from both sets of trial experiments (Tables 2 and 3) showed

Table 1
Recovery efficiencies (%) of OSCS and GAGs of contaminated HP drug product using P-6 column eluted with 0.5 M NaCl or 0.5 M NH_4HCO_3.

	OSCS[a]		Contaminated HP samples 7–12 (eluted with NH_4HCO_3)
	Eluted with NaCl	Eluted with NH_4HCO_3	
Loading (mg)	2.1 ± 0.3[b]	2.6 ± 0.3[b]	54.8 ± 0.3[c]
Recovery (mg)[d]	2.2 ± 0.3[b]	2.6 ± 0.3[b]	41.5 ± 1.0[c]
Recovery efficiency (%)	105	100	–

[a] No salt is present in OSCS sample.
[b] Average value (wt) ± standard error of duplicate experiments.
[c] Average value (wt) ± standard error of sextuplicate experiments.
[d] Recovery of OSCS was determined using carbazole assay.

Table 2
Mass balance of GAGs of artificial mixtures of HP, OSCS, and DS (samples 1 and 2) and contaminated HP (samples 7–9) obtained following desalting using P-6 column eluted with 0.5 M NH_4HCO_3 and digestion with heparin lyases I, II, and III.

		Artificial mixture		Contaminated HP drug product[a]
		1	2	7–9
GAG Amount loaded (mg)	HP	32.5	24.1	41.5 ± 1.0
	DS	5.0	3.3	
	OSCS	4.6	8.3	
Amount recovered (mg)	DS + OSCS	9.2	11.1	11.0 ± 0.3
Recovery efficiency (%)	DS + OSCS	95.8	95.7	–
Calculated mass of HP (mg)		–	–	30.5 ± 0.5

[a] Average values (wt) ± standard deviations of triplicate experiments.

Table 3
Mass balance of GAGs of artificial mixtures of HP, OSCS, and DS (samples 3 and 4) and contaminated HP (samples 10–12) obtained following desalting using P-6 column eluted with 0.5 M NH_4HCO_3 and digestion with heparin lyases I, II, and III and chondroitinase ABC.

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

[a] Average values (wt) ± standard deviations of triplicate experiments.

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 (Tables 2 and 3) showed that there was 9.3 ± 0.1 mg of GAGs, which was completely resistant to heparin lyase and chondroitin lyase digestion. Furthermore, each vial of contaminated formulated HP drug product contained 1.7 ± 0.3 mg of DS and 30.5 ± 0.5 mg of HP. Chromatographic resolution of OSCS and HP disaccharides on the P-6 column was excellent (Fig. 3).

NMR analysis on the recovered OSCS

The recovered OSCS from artificial GAG mixture samples and the intact heparin and chondroitin lyase-resistant GAGs isolated from the contaminated HP drug product were analyzed with ^{1}H NMR and 2D NMR (^{1}H NMR and HMQC [see Fig. 4E and Table S3 in supplementary material], ^{1}H–^{1}H COSY [see Fig. S2 in supplementary material]). The signals of intact GAGs from the contaminated HP drug product, samples 10–12, were identical to the signals of OSCS from artificial mixtures 3 and 4, confirming that the GAG contaminant consisted of entirely OSCS. All signals of GAGs from the contaminated HP drug product, samples 7–9, 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 GAGs from the contaminated HP drug product were analyzed using PAGE (Fig. 5). The OSCS was polydisperse with a range of MWs from 7.3 to 60.5 kDa and a MW_{avg} of 31.1 kDa based on the HP oligosaccharide standards.

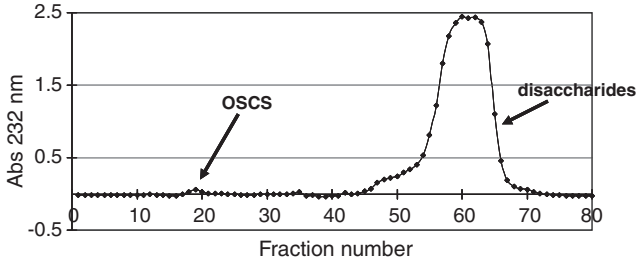

Fig.3. Chromatography on a P-6 column of contaminated HP drug product after processing. Contaminated HP drug product was desalted using a P-6 column eluted with NH_4HCO_3, and recovered GAGs were freeze-dried, added to phosphate-buffered saline, and treated with heparin lyases (I, II and III) and chondroitinase ABC. Enzymes were removed using an SCX spin column, and the sample was analyzed on the P-6 column with UV detection at 232 nm. Peaks corresponding to OSCS and HP disaccharides are indicated.

Disaccharide compositional analysis

HP disaccharide composition was analyzed by HPLC–ESI–MS to provide further information on the purity of HP in the sample (Fig. 6A). The percentages of the major disaccharide, ΔUA2S-GlcNS6S (in extracted ion chromatogram [EIC] and UV), of HP recovered from the artificial mixture and contaminated HP drug product sample were 88.0% and 83.7%, respectively. In addition, other disaccharides, including ΔUA-GlcNS, ΔUA-GlcNAc6S, ΔUA-GlcNS6S, ΔUA2S-GlcNS, and ΔUA2S-GlcNAc6S, were detected. All of the peaks in the EIC spectrum were identified from their corresponding MS spectrum.

DS disaccharide composition was also analyzed by HPLC–ESI–MS (Fig. 6B). The disaccharide composition profile (major and minor disaccharides) of DS isolated from contaminated HP drug product sample was well matched with the commercial DS from Celsus Laboratories. The percentages of the major disaccharide, ΔUA-GalNAc4S, 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 2 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 [16,38]. 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 MW_{avg} of its CS precursor as well as the completeness of its chemical sulfonation [7]. The small amount of OSCS contaminant (usually reported at 1–25% [40]) relative to HP makes its isolation and identification difficult. OSCS also can inhibit heparin and chondroitin lyases [45]; thus, care must be taken to use sufficient amounts of highly active enzymes in the analysis of 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 and 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. Two unopened boxes containing 25 × 1-ml vials (lot 117050, exp. 11/2009), with each vial containing 5000 USP U/ml (Fig. 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 were provided on a vial-by-vial basis to analysts in our laboratory for the current studies.

The initial GAG qualitative assessment on the contaminated HP drug was conducted with CE and SAX–HPLC by using the conditions from Refs. [21] and [30]. In CE analysis, the anions and cations migrate in different directions, but they are all swept toward 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 the highest negative charge was eluted first, followed by HP and then DS

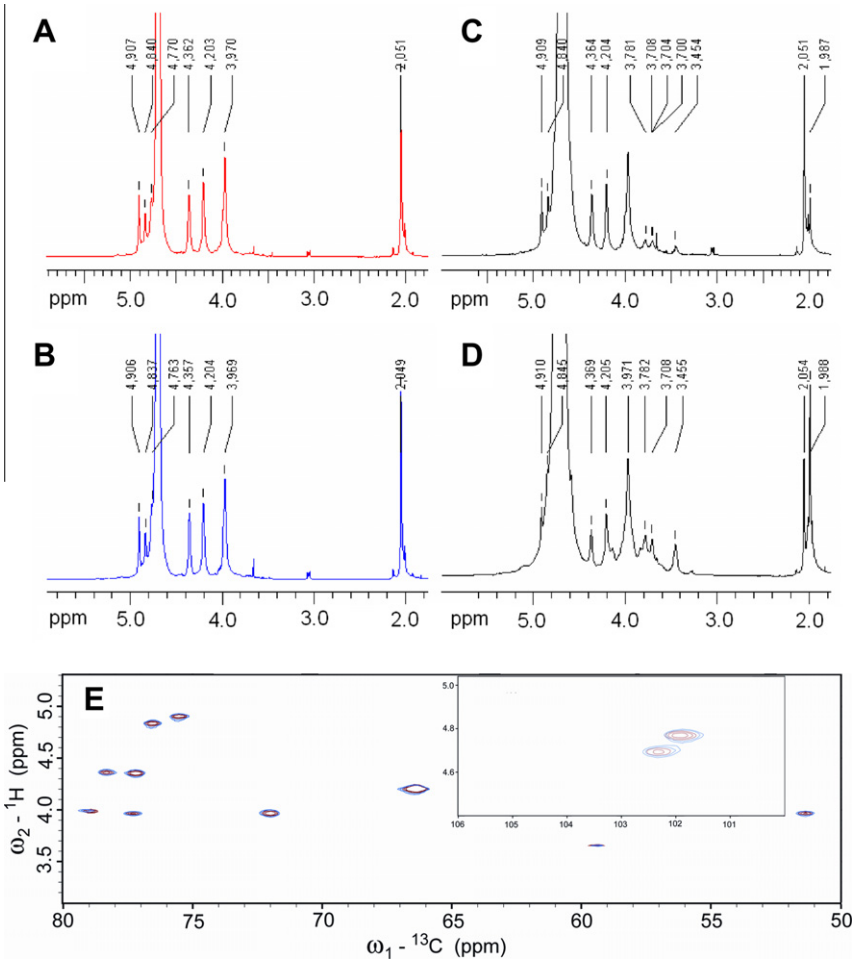

Fig.4. NMR spectra (600 MHz) of GAG samples. (A) Proton NMR spectrum of OSCS recovered from contaminated HP drug product. HP and DS were removed by complete digestion with heparin lyases and chondroitinase ABC. (B) Proton NMR spectrum of OSCS recovered from artificial mixture of HP (33.9 mg), OSCS (4.5 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}H–^{13}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). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(see Fig. 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 the lowest negative charge was eluted first, followed by HP and then OSCS (see Fig. 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 coinjection experiments (Fig. 2A and B). Unfortunately, the peaks were insufficiently resolved and too broad to obtain accurate integration. 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 of benzyl alcohol, and added NaOH and HCl to adjust pH to 5.0–7.5, 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 [boiling point = 208 °C] remains). The observed mass was 54.8 ± 0.3, consistent with the presence of additional components (i.e., impurity and/or contaminant) in the API. Next, a series of control experiments were used to ensure the removal of the NaCl (MW = 58 g/mol) and benzyl alcohol (MW = 108 g/mol) excipients. Because of their low MWs compared with GAGs in the API, our initial focus was on size separation. Three different methods (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) showed a variable recovery efficiency of 77.5–90.5%, with a higher recovery for the higher MW and higher charged OSCS than for HP. The P-2 column also had some drawbacks for the quantitative removal of salt (Table S2), showing recovery efficiencies of 82.8% for OSCS and 97.0% 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 GAGs. The adsorption of OSCS on the P-2 column further retarded its elution. Thus, simple desalting of the 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 NH_4HCO_3 provided the optimal method to both desalt the sample and separate small molecules of MW < 6000 (i.e., salt, benzyl alcohol, and disaccharides)

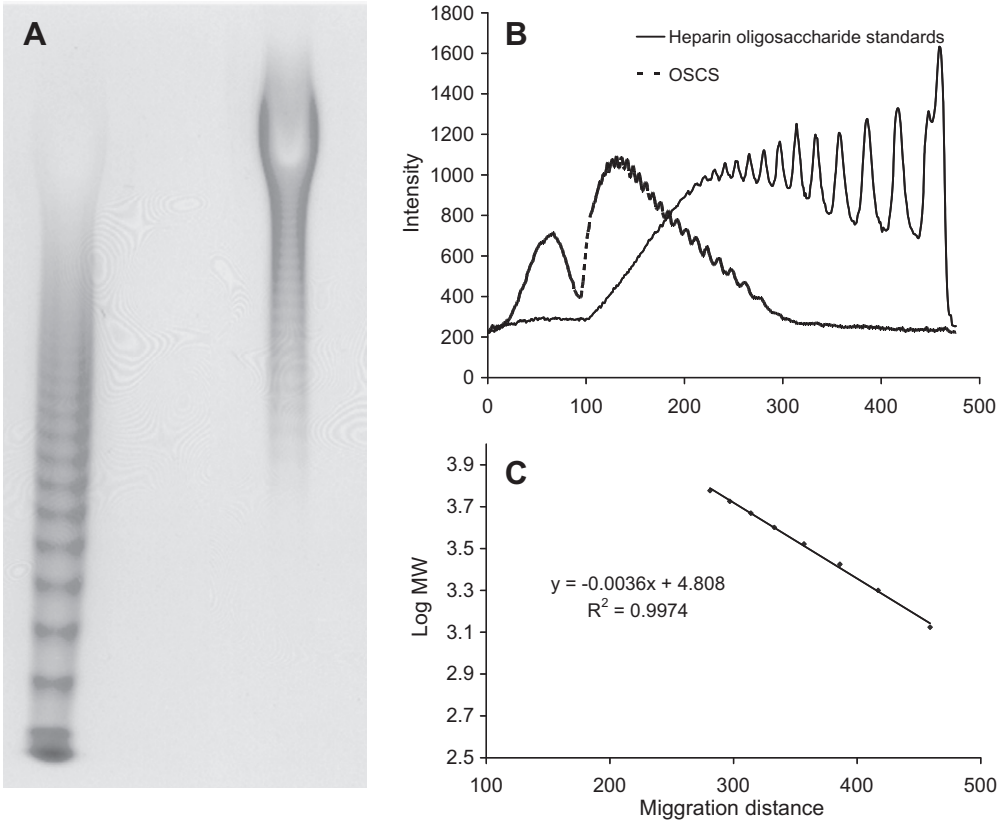

Fig.5. PAGE analysis of OSCS recovered from contaminated HP drug product. (A) Left lane: HP oligosaccharide standards; right lane: recovered OSCS. (B) Band intensities of HP oligosaccharide standards and recovered OSCS. (C) Plot of log MW as a function of migration distance (mm) used to estimate the MW of OSCS contaminant.

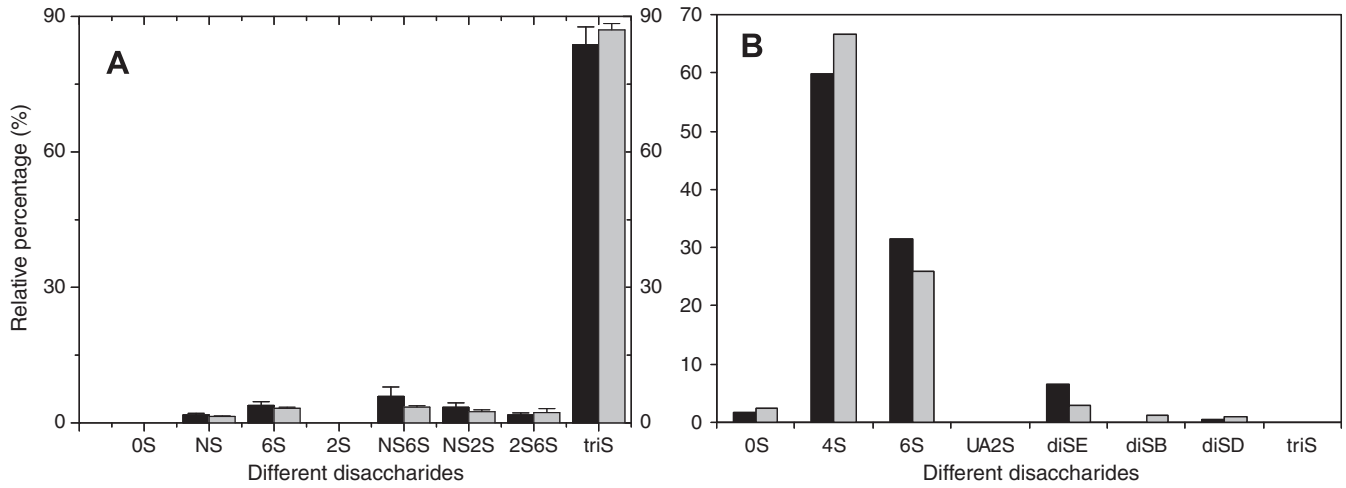

Fig.6. (A) Disaccharide composition of HP recovered from contaminated HP drug product (black bars) and disaccharide composition of HP recovered from an artificial mixture of HP, OSCS, and DS (gray bars). (B) Disaccharide composition of DS recovered from contaminated HP drug product sample (black bars) and commercial porcine intestinal DS from Celsus Laboratories (gray bars).

from GAGs. 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 higher than 95% (the total recovery efficiency after the processes of enzyme digestion, deproteination, and separation). From 54.8 ± 0.3 mg of freeze-dried contaminated HP drug product, 41.5 ± 1.0 mg of GAGs 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 toward GAGs, and the chondroitin lyases were purified from organisms that do not contain heparin lyases [46]. Thus, these enzymes represent ideal tools to determine GAG composition. There have been few articles reporting the analysis of contaminated HP drug product sample using enzymatic digestion because the impurity and contaminants

(DS and OSCS) reportedly inhibit the heparin lyases. However, this inhibition is competitive and reversible; thus, prolonged treatment (72 h) with highly active heparin lyases at 31–37 °C in sodium phosphate–NaCl buffer can digest HP completely [37]. Heparin lyases and chondroitin lyases (cationic proteins), used to treat the 41.5 ± 1.0 mg of recovered GAGs, were removed by cation exchange using a Vivapure MAXI SH column and the separation on a P-6 column to afford 11.0 ± 0.3 of non-HP GAGs and 9.3 ± 0.1 mg of non-HP, non-DS/CS GAGs. These data indicate that a single vial contains 30.5 ± 0.5 mg of HP (heparin lyase digestible), 9.3 ± 0.1 mg of contaminating GAGs (not digestible by heparin and chondroitin lyases), and 1.7 ± 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}H NMR, HMQC, and HHCOSY) (Fig. 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., δ_H = 3.02 and 3.44–3.63 ppm) corresponding to impurities assigned to residual ammonium bicarbonate were the same in each sample. The MW_{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 mixtures 1 and 2 at δ_H values of 1.99, 3.46, and 3.71–3.78 ppm are of greater intensity than those in the contaminated HP drug product samples 7–9 because of the larger amount of DS in the artificial mixtures (3.7 and 4.2 mg in artificial mixtures 3 and 4, respectively) than in the contaminated HP drug product sample (1.7 ± 0.3 mg).

HPLC–MS was applied to differentiate HP from HS because HS was another potential impurity of HP product and can also be degraded by heparin lyases. Because HP is primarily highly sulfated, with approximately 60–90% of the disaccharide units consisting of →4) IdoA2S (1→4) GlcNS6S (1→, on heparin lyase treatment it should give rise to primarily (∼60–90%) ΔUA2S (1→4) GlcNS6S (TriS). In contrast, HS is less sulfated; with approximately 60–90% of the disaccharide units consisting of →4) β-D-GlcA (1→4) β-D-GlcNAc, on heparin lyase treatment it should give rise to primarily (∼60–90%) ΔUA (1→4) GlcNAc (0S). Disaccharide analysis of the contaminated HP drug product samples 7–12 isolated using the P-6 column was measured (Fig. 6A) showed 83.7% TriS disaccharide unit with no observable 0S disaccharide, confirming that there was no detectable HS present in the contaminated HP drug product. Although we cannot absolutely rule out the presence of any other contaminants or impurities (at levels below a few weight percentage points) 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 (Fig. 2) as determined by carbazole assay (having a limit of detection <1 μg). Furthermore, our NMR analysis of recovered GAGs shows no evidence of signals characteristic [10,38] of either intact or fragmented oversulfated DS, HP, or HS.

In conclusion, a mass balance analysis of a known contaminated HP product has been performed. On freeze-drying, the contaminated vial contained 54.8 ± 0.3 mg of dry weight composed of 11.4 ± 0.5 mg of NaCl and 0.9 ± 0.5 mg of residual benzyl alcohol (added excipients), 30.5 ± 0.5 mg of HP, 9.3 ± 0.1 mg of OSCS, and 1.7 ± 0.3 mg of DS. No other contaminants or impurities were detected. The detection limit of the primary assay used to close mass balance, weighing ±0.1 mg on approximately a 10-mg sample, is insignificant in our mass balance analysis compared with 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 be prospectively applied to determine many other types of potential contaminants present in HP.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.09.015.

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